University of Alberta

Lactose hydrolysis by disrupted thermophilic lactic acid bacteria

by

Todor Vasiljevic

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Lactose Hydrolysis by Disrupted Thermophilic Lactic Acid Bacteria submitted by Todor Vasiljevic in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Science and Technology.

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Dedicated to my family
Ivana, Dejan and Danijela

Abstract

A novel process for the lactose hydrolysis in dairy systems was evaluated from the technical feasibility standpoint, using β-galactosidase-containing crude cellular extracts (CCE) from a mechanically disrupted culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (LB11842).

The maximization of β-galactosidase (β-gal) activity was attempted by optimizing growth conditions of LB11842 cultivation. Enrichment of whey or whey permeate based media with whey protein preparations, yeast extract or MRS slightly improved culture growth and β-gal activity, but was inferior to cultivation in sterile skim milk. In contrast to NaOH or KOH, which had a similar effect, the NH_4OH improved biomass production and β-gal activity with concomitant proteolytic activity suppression and enhancement of exopolysaccharide formation.

The CCEs from three potential sources of the β-gal activity, LB11842, *Lactobacillus delbrueckii* ssp. *lactis* DMF 3078 and *Streptococcus thermophilus* 143 (St143), were compared for lactose hydrolysis, as well as for transferase and proteolytic activities. The highest lactose hydrolysis rate and proteolytic activity were achieved using LB11842. Different neutralizers had no effect on the rate constant k_{cat} which was significantly different ($p \leq 0.05$) among the CCEs, with temperature dependence following the Arrhenius kinetics. St143 CCE exerted significantly ($p<0.05$) higher transferase activity. The maximum oligosaccharide formation by all three CCEs was around 50°C. Maximum proteolytic activity, using the response surface methodology, was predicted to be around 43°C.

The preservation of β-gal activity in LB11842 CCE preparations by spray- or freeze-drying was a function of process conditions and type of adjuncts. The presence of lactose was essential for almost complete activity preservation. Enzyme activity after spray drying was highly dependent on inlet temperature and residual moisture after drying.

Addition of 2 or 4% CCE to skim milk resulted in increased sweetness as well as noticeable off-flavors, probably carried over from the β-gal production step. Use of 1% KOH CCE resulted in the products being closest to the control skim milk, while the NH_4OH imparted grossly detrimental taste. The proposed lactose hydrolysis process using the CCE technology appeared to be technically feasible in selected dairy systems, while other possible applications of the CCE approach were also identified.

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Chapter 1

Introduction and Objectives

1.1. Introduction

The first sugar humans and other mammals encounter in their lives is O-β-D-galactopyranosyl-(1->4)-β-D-glucopyranose, also known as lactose. This disaccharide is a major component of milk but otherwise it rarely occurs in nature. Of all mammals, human milk contains the highest amount of lactose, 6.7% on an average, compared to bovine milk with 4.9% lactose (Holsinger, 1997). Following ingestion, lactose is hydrolyzed to two monosaccharides by the enzyme β-galactosidase in the small intestine (de Vrese et al., 2001). The products of the lactose hydrolysis, glucose and galactose, are actively absorbed into the blood stream. However, a decline in human intestinal β-galactosidase activity during midchildhood may result in a gradual loss of the ability to utilize the lactose, a condition known as lactose maldigestion or lactose intolerance (Lee and Krasinski, 1998). This decline is prevalent in certain populations, affecting mainly non-caucasians (Shah and Jelen, 1989; Sahi, 1994). Lactose intolerance leads to self-imposed dietary restriction of dairy products, which in turn may have deleterious nutritional effects in later life. On the other hand, lactose intolerant individuals may consume yogurt and perhaps other fermented dairy products containing live organisms of lactic acid bacteria (LAB) with little or no symptoms of lactose maldigestion and intolerance. This is likely due to the action of intracellular β-galactosidase, released upon the LAB cell lysis in the gut, resulting in an enhancement of the lactose hydrolysis in the small intestine (Mustapha et al., 1997).

High prevalence of lactose intolerance in the world prompted research on an industrially suitable methodology for lactose hydrolysis that would result in a wider range of readily consumable lactose-containing dairy products. An industrially applicable lactose hydrolysis process could also reduce technological problems associated with lactose crystallization (Zadow, 1993), as well as open new possibilities for the production of value-added dairy products, nutraceuticals and health ingredients from dairy sources, including the underutilized whey.

Lactose may be hydrolyzed either by strong acids or enzymatically. The strength of the lactose glycosidic bond requires fairly harsh processing conditions during acid hydrolysis that may result in secondary reactions such as formation of certain compounds through Maillard reaction, caramelization, detrimental flavour effects as well as equipment corrosion. An alternative to acid hydrolysis is the use of β-galactosidases, enzymes identified in numerous sources (Agrawal et al., 1989). Commercial β-galactosidase preparations have been generally derived from microbial sources (Mahoney, 1997). Industrially, enzymatic lactose hydrolysis in dairy-based systems can be performed by three major approaches: direct addition of soluble lactase preparations; direct addition with subsequent enzyme recovery by ultrafiltration; or using enzyme immobilization technology. However, the extra cost associated with the purification and single-use during production of lactose-hydrolyzed products and/or equipment investment costs, may render these approaches economically questionable.

To avoid high cost, Jelen (1993) suggested the possibility of using a sonicated culture of dairy lactic acid bacteria for lactose hydrolysis. The process involves "in-house" cultivation of a high β-galactosidase-producing strain of a commonly used dairy

starter culture, followed by cell collection and disintegration. The enzyme was liberated by high pressure homogenization, a process readily available in the dairy industry. The disrupted culture was then added to suitable substrates such as skim milk, whey or other suitable material. Recently completed studies (Kreft, 2001; Bury, 2000) commented on several aspects of this approach and indicated no major problems.

1.2. Objectives

The overall aim of this Ph.D. project was an in-depth exploration of the technical feasibility of the lactose hydrolysis process using mechanically disrupted cultures of dairy lactic acid-producing bacteria and to investigate some of its main components with additional potential applications of the crude cellular extract (CCE) approach.

The specific objectives of the current work were: 1) to design a feasible lactose hydrolysis process based on in-house production of the β-galactosidase from *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842; 2) to investigate the effects of some environmental conditions on the growth characteristics and β-galactosidase activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842; 3) to explore selected enzymatic activities during the lactose hydrolysis by β-galactosidase-containing crude cellular extracts from selected dairy starter cultures in dairy based systems; 4) to evaluate the effects of different drying methods on the retention of the β-galactosidase activity in crude cellular preparations upon drying and storage; and 5) to provide a cursory assessment of the sensory impact of lactose hydrolysis by crude β-galactosidase preparation in selected dairy systems.

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Chapter 2

Literature review

2.1. Lactose

Lactose, the major carbohydrate in milk of most mammals, usually occurs free, but small amounts appear in the form of the lactose containing oligosaccharides as well. The constitutive monosaccharides of lactose are galactose and glucose, joined through β-1, 4 glycosidic bond (Figure 2-1). The concentration of free lactose varies in mammalian milk, depending on the species, i.e. human and cow milk contain on average 6.7 and 4.9%, respectively. The principal advantage of lactose over its constitutive monosaccharides is the provision of the higher caloric value to young mammals for the same given osmotic pressure (Holsinger, 1997).

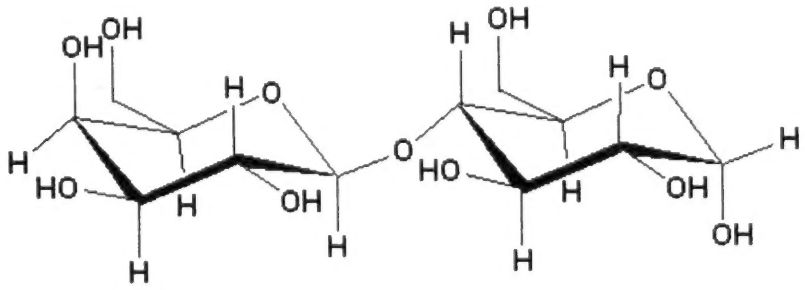

Figure 2-1: α-lactose

Lactose is present in milk as an equilibrium of two anomeric forms: α and β, designated by the position of the hydroxyl group on the reducing end. Regardless of the lactose conformation, mutarotation allows one form to pass readily into the other, when a solution of either is formed. The distribution of α and β anomers in an aqueous solution

of lactose is about 37.3% α and 62.7% β at 20°C. The α and β forms show distinctly different solubilities. The true solubility of α form is ~ 7 g/100 g of water, while that of β form is ~ 50 g/100 g of water. Due to mutarotation, the equilibrium of the two forms is reached at the final solubility of lactose in water which is ~ 17 g/100 g of water, 10 times less than that of sucrose (Jelen, 1985). Although the solubility of lactose is low compared to other sugars, lactose solutions may become highly supersaturated before spontaneous crystallization occurs. The type of the crystalline lactose anomeric form depends on the processing temperature applied during the concentration step for saturation of a lactose solution. The anhydrous β-lactose is crystallized at temperatures above 93.5°C, as opposed to α-form crystallizing below this temperature (Holsinger, 1997). The crystals of α-lactose are hard, poorly soluble, and when the crystal size exceeds about 16 μm, "sandiness", a texture defect, results. This defect can mainly occur in concentrated dairy products such as sweetened condensed milk, ice cream and whey cheese. Sandiness may be controlled by enzymatic hydrolysis of lactose by the enzyme β-galactosidase, which hydrolyzes lactose into its constitutive monosaccharides with greater solubility in water than lactose (Holsinger and Kligerman, 1991). The sweetness of a 10% lactose solution is about 20% that of sucrose at the same concentration. On the other hand, glucose and galactose are 75 and 70% as sweet as sucrose, respectively. Thus, lactose hydrolysis greatly enhances the overall sweetness perception in the final product (Horton, 1993).

2.2. Lactose maldigestion and lactose intolerance

The decline of the intestinal β-galactosidase (β-gal) activity is a biological characteristic of the maturing intestine in the majority of the world's human population

(Lee and Krasinski, 1998) but also affects other mammals (Zadow, 1993). Upon ingestion, lactose is hydrolyzed by β-gal in the brush border membrane of the mucosa of the small intestine into constitutive monosaccharides, which are readily absorbed in the blood stream (Shah and Jelen, 1991; de Vrese et al., 2001). However, the activity of the intestinal β-gal in lactose intolerant individuals decreases to less than 10% of that found during childhood (Buller and Grand, 1990). This decline, termed hypolactasia, causes insufficient lactose digestion in the small intestine, resulting in a condition designated as lactose maldigestion, usually determined by either lack of an increase in blood glucose concentration or increase in breath hydrogen concentration upon digestion of 50 g lactose (Scrimshaw and Murray, 1988).

There are several types of hypolactasia: primary, demonstrated in an autosomal recessive pattern of inheritance regulated by the rate of β-gal gene transcription (Lloyd et al., 1992); secondary, due to inflammation of the small intestine; and tertiary, congenital lactose malabsorption, a rare autosomal-recessive heritable genetic defect evident immediately after birth (Johnson et al., 1993).

The lactose maldigestion, also called β-gal nonpersistence, is prevalent in the majority of the world's population. With the exception of the inhabitants of Northern and Central Europe and Caucasians in North America and Australia, over 70% of adults worldwide are unable to digest lactose (de Vrese et al., 2001). Hypolactasia and lactose maldigestion accompanied by clinical symptoms, such as bloating, flatulence, nausea, abdominal pain, and diarrhea, are termed lactose intolerance. Symptoms are caused when lactose passes through the large intestine undigested, reaching the small intestine where it is fermented by intestinal microflora. Produced metabolites change the environmental

osmotic pressure resulting in increased water flow into the lumen. The severity of the symptoms depends primarily on the size of the lactose load ingested (Savaiano and Levitt, 1987). The development of symptoms the intolerance also depends on the rate of lactose transit to the large intestine, which is influenced by the osmotic and caloric load, and the ability of the colonic microflora to ferment lactose (Martini and Savaiano, 1988; Johnson et al., 1993). Whole and chocolate milk are better tolerated than skim milk or lactose solution in water (Leichter, 1973; Welsh and Hall, 1977). Lactose intolerance usually leads to self-imposed dietary restrictions of dairy products, which in turn may have deleterious nutritional consequences in later life, such as low calcium absorption resulting in osteoporosis (Pietschmann et al., 1991). Numerous approaches have been evaluated in the development of products that would enable β-gal deficient individuals to consume products of dairy origin. Generally, there are two approaches: the consumption of fermented products, such as yogurt, which have been shown to be well tolerated by lactose intolerant persons (Kolars et al., 1984; Martini et al., 1987a; Martini et al., 1987b; de Vrese et al., 2001), or the use of food-grade β-galactosidase preparations or similar processes to pre-hydrolyze lactose in dairy products.

2.3. Methods for lactose hydrolysis

2.3.1. Lactose hydrolysis by acids

Lactose may be hydrolyzed either by strong acids or enzymatically. When disaccharides are heated in the presence of acidic moieties and water, one water molecule is added to the glycosidic linkage and two monosaccharides are formed. The strength of the glycosidic bond determines the conditions required for this reaction. Lactose,

compared with other disaccharides, is relatively stable to acid hydrolysis, thus requiring very severe pH (1 to 2) and temperature (100 to 150°C) conditions for the successful cleavage of the lactose β-bond.

Despite these chemical characteristics of the lactose β-bond, several approaches have been tested for the production of lactose-hydrolyzed syrups from whey and whey permeate using strong mineral acids (Coughlin and Nickerson, 1975; Lin and Nickerson, 1977; Vujicic et al., 1977; Hartofylax et al., 1989; Huh et al., 1990). Organic acids are not effective in cleaving the lactose (Holsinger and Kligerman, 1991). The rate of lactose hydrolysis in whey by acid solutions is a function of time, acid concentration and reaction temperature. Increasing any of these parameters results in an acceleration in the rate of hydrolysis. By optimizing all the process parameters, over 90% of the original lactose can be hydrolyzed (Zadow, 1986). The presence of nitrogenous compounds and minerals in whey results in secondary reactions that affect the purity of the lactose-hydrolyzed syrup. Some of the secondary reactions include formation of oligosaccharides, caramelization, and in extreme cases, charring of the product. The presence of non-protein nitrogen promotes the development of the dark color through Maillard reaction (Holsinger, 1997). Generally, secondary reactions are one of the reasons for the limited industrial use of acid hydrolysis for lactose hydrolysis.

As an alternative to the addition of mineral acid, the process for lactose hydrolysis utilizing a strong acid cation exchange column was devised (Macbean et al., 1979). Approximately 80% of lactose was hydrolyzed in 3 min at 150°C and pH 1.2 using this process, and the syrup that was obtained was demineralized and decolorized. The

advantages of this process are that it may be used continuously with resin regeneration and reduced costs.

The major disadvantage of all of these proposed approaches to lactose hydrolysis is their technical impracticality for use in certain industrial applications, such as lactose hydrolysis in milk. The formation of undesirable products by side-reactions introduces necessity for the introduction of an additional purification step (de Boer and Robbertsen, 1981) and the low pH – high temperature conditions, necessary for a high rate of acid lactose hydrolysis, require specific materials capable of withstanding prolonged use in such environment, thus incurring additional cost (Gekas and Lopez-Leiva, 1985).

2.3.2. Enzymatic lactose hydrolysis

An alternative to acid hydrolysis of lactose is the use of the enzyme β-galactosidase (E.C. 3.2.1.23, β-gal), which can be employed in a number of ways to hydrolyze lactose in dairy products. The choice of process technology will be dictated by the nature of the substrate, enzyme characteristics and the economics of the final product. Generally, there are three major approaches to enzymatic lactose hydrolysis in dairy products: 1) batch processing; 2) enzyme recovery by ultrafiltration; and 3) immobilized enzyme technology.

The batch processing involves the addition of highly purified enzyme to a dairy product and holding the mixture at a specified temperature until the desired degree of hydrolysis is reached, whereupon the lactose-hydrolyzed product is pasteurized and packaged. Alternatively, sterile enzyme may be added into sterilized milk under aseptic conditions and the desired degree of hydrolysis achieved during storage. Seemingly easy

and efficient, the batch process is costly, since the expensive preparation of β-gal must be used in a "throw-away" mode. The β-gal should be highly purified to eliminate the contamination of milk with proteases, which may compromise the quality of the final product (Modler et al., 1993). Commercial preparations of β-gal, such as Lactaid and Lacteeze in the form of solution or tablets, are also available for individuals suffering from lactose intolerance. However, the cost of these preparations is even higher in comparison to lactose hydrolyzed milk (Mahoney, 1997).

To decrease the cost associated with the enzyme loss, enzyme recycling utilizing high flux ultrafiltration has been proposed by Miller and Brand (1980). This approach is based on the ease with which lactose and its hydrolysis products permeate through ultrafiltration membranes; in contrast, the enzyme is captured by the membrane in the retentate. Based on this relatively simple approach, several processes have been proposed and tested in whey (Foda and Lopez-Leiva, 2000) or UHT milk (Splechtna et al., 2002). The operating life of the membrane based processing system for the lactose hydrolysis was mainly limited by the enzyme instability due to binding onto a membrane surface and concomitant irreversible loss of the enzyme catalytic activity (Splechtna et al., 2002).

The cost of the hydrolysis can be decreased further by employing immobilization techniques such as adsorption, entrapment and covalent bonding of the enzyme to a suitable carrier (Greenberg and Mahoney, 1981; Gekas and López-Leiva, 1985; Baran et al., 1997; Eldin et al., 2000; El-Masry et al., 2000). Lactose hydrolysis in skim milk by immobilized technology poses several problems such as adsorption of milk proteins onto a synthetic support and/or microbial contamination, thus the system should be regenerated and sterilized regularly (Honda et al., 1993; Splechtna et al., 2002). On the

other hand, lactose hydrolysis in whey encounters fewer problems and several commercial systems have been commissioned. One of them was the Corning process, developed by the Corning glass company (Corning, NY, USA), employing the covalently bound β-gal from *Aspergillus niger* onto glass beads (Mahoney, 1997). In comparison to the Corning process, the Valio Laboratory has developed a system for lactose hydrolysis in demineralized whey or whey permeate utilizing the adsorption of β-gal onto a phenol-formaldehyde resin (Mahoney, 1997). The enzyme remains active for several years under normal processing conditions. Generally high enzyme instability, low lactose conversion rate and initial investment costs are among the reasons responsible for the fact that the Valio hydrolysis process to be the only one currently operational on the industrial scale.

As an alternative to all of these processes, Jelen (1993) suggested that the substantial cost associated with the β-gal purification and maintenance could be reduced by use of sonicated or otherwise disrupted culture of high β-gal producing strains of lactic acid bacteria. Lactic acid bacteria (LAB) play an important role in the production of fermented products, utilizing lactose as a carbohydrate source. While the amount of lactose converted into lactic acid during the fermentation is not great, usually less than 30%, lactose utilization could be enhanced by β-gal liberation from the cell using sonication. Using this method, Toba et al. (1990) hydrolyzed 71 to 74% of lactose in milk in comparison to unsonicated culture, which was shown to hydrolyze only 39%.

2.4. Sources of β-galactosidase

Numerous β-gal sources are found in nature (Agrawal et al., 1989); however, only the enzyme of microbial origin has been exploited commercially (Mahoney, 1997). The

industrially utilized β-gal is derived from yeasts such as *Kluyveromyces* (*K.*) *lactis* and *K. fragilis* or fungi such as *Aspergillus* (*A.*) *niger* and *A. oryzae* (Holsinger and Kligerman, 1991) and some bacteria such as *Bacillus circulans*. These enzymes differ widely in their properties, especially in their pH optima; *K. lactis* β-gal has a pH optimum in the neutral range with the temperature optimum about 35°, while β-gal from *K. fragilis* has a pH optimum at 4.8 and a temperature optimum above 50°C (Holsinger and Kligerman, 1991). These properties limit the use of a certain enzyme for a particular operation. More recently, the attention has been focused on the properties of the β-gal derived from LAB, primarily for their GRAS (Generally Regarded As Safe) status (Stiles and Holzapfel, 1997).

2.5. Lactic acid bacteria

Lactic acid bacteria (LAB) are typically described as Gram-positive, non-motile, non-spore forming, catalase negative cocci or rods. They are chemo-organotrophic, grow only on complex media and produce lactic acid as the major end product during sugar fermentation (Schleifer and Ludwig, 1995). The term LAB usually refers to certain species of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* with newly proposed groupings (Stiles and Holzapfel, 1997). Phylogenetically, they belong to the clostridial branch of the gram-positive bacteria with G + C (guanine + cytosine) content less than 55 mol% in their DNA. Growing on glucose, LAB may be either homofermentative, producing more than 85% lactic acid, or heterofermentative, producing lactic acid, carbon dioxide, ethanol and/or acetic acid in equimolar amounts (Hammes and Vogel, 1995). Sugar can be imported into the cell by three fundamentally different transport systems: group translocation, primary transport and secondary

transport systems (de Vos and Vaughan, 1994). Bioenergetically, the most efficient system is the sugar-specific phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS), in which sugar is phosphorylated during transport. Primary transport systems are widespread and involve a sugar transport ATPase, belonging to the superfamily of ATP binding cassette (ABC) proteins. On the other hand, sugar translocation in secondary transport systems is coupled to ions or other solutes (de Vos and Vaughan, 1994). After the sugar is imported inside the cell, it is phosphorylated and metabolized via glycolysis, also known as Embden-Meyerhof-Parnas (EMP) pathway, to pyruvate in homofermentative LAB or via 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway in heterofermentative LAB (Cocaign-Bousquet et al., 1996). In homofermentative LAB, pyruvate is further reduced mainly into lactic acid to regenerate reduced coenzymes involved in the glycolytic pathway, thus maintaining the energetic equilibrium.

2.5.1. Lactose metabolism

The importance of LAB in the dairy industry for the production of fermented products has led to extensive research on the lactose metabolism of LAB. Lactose is imported into the cell either via the PEP-PTS or by secondary transport systems. Primary transport systems for the lactose translocation have not been characterized in LAB so far (de Vos and Vaughan, 1994). Lactose translocated via PEP-PTS system is phosphorylated during the transport in the cell wall and, when inside the cell, cleaved by phospho-β-galactosidase. Resulting glucose is metabolized by enzymes of the glycolytic pathway, and galactose is converted into tagatose and cleaved into trioses, entering the glycolytic pathway (Cocaign-Bousquet et al., 1996). The presence of PEP-PTS system, a

plasmid-mediated trait, is a very important characteristic of dairy LAB used in cheesemaking for rapid lactic acid production. In contrast, lactose imported by a secondary transport systems is first cleaved in the cytoplasm by β-galactosidase (Cocaign-Bousquet et al., 1996); the glucose will enter glycolysis, while the utilization of galactose depends on the presence of enzymes of the Leloir pathway (Figure 2-2).

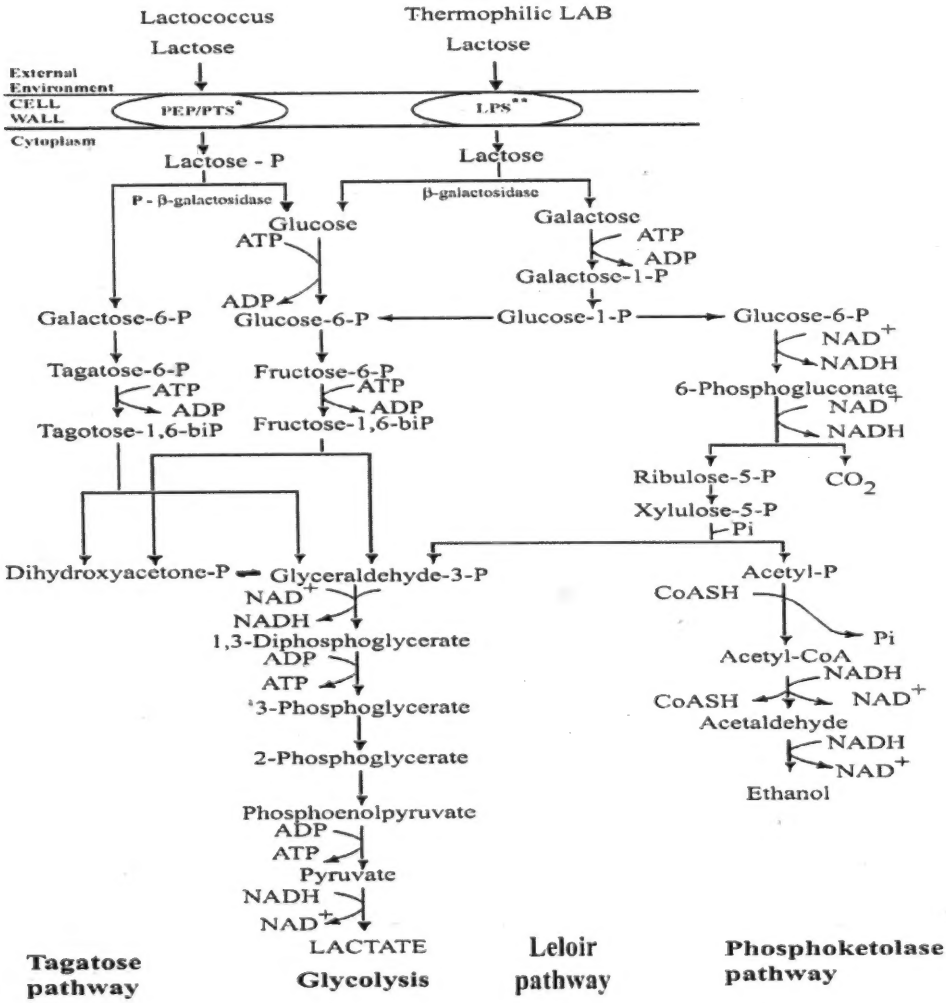

Figure 2-2: Lactose metabolism in Lactic Acid Bacteria
(*group translocation PEP/PTS system; **lactose permease import system; adapted from various sources)

2.5.2. Exopolysaccharide production

Some of the dairy LAB cultures are capable of producing extracellular polysaccharides (EPS) (Cerning, 1995). The polysaccharides produced are either homopolysaccharides (Cerning, 1995), e.g. glucans and fructans, or heteropolysaccharides (Stingele et al., 1996). Several of these EPS have important functional properties and determine the viscosity of dairy products including yogurt and specific Scandinavian fermented milk products, such as viili and longfil (Neve et al., 1988). The strain, the culture conditions and the medium composition affect the amount of EPS produced by certain species (Degeest and de Vuyst, 1999; Petry et al., 2000). The type of carbon source has a huge influence on EPS productivity and may also affect the composition of EPS. *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2772 produced three times more EPS with glucose than with fructose as a sugar source, and the type of EPS produced by this organism was influenced by the sugar source as well (Grobben et al., 1997). The amount and composition of EPS was also strongly affected by carbon/nitrogen ratio in the growth medium (Degeest and de Vuyst, 1999).

Biosynthesis of EPS produced by LAB includes the intracellular formation of EPS precursors, the sugar nucleotides, followed by the formation of a repeating unit on a lipid carrier, which is located in the cytoplasmic membrane (Figure 2.3). The last step of the EPS formation involves transport of the repeating units across the membrane to the outer layer and polymerization of several hundred to several thousand repeating units to form the final EPS (Ramos et al., 2001). The repeating unit in the EPS from *Streptococcus thermophilus* is a tetrasaccharide consisting of galactose and glucose (Doco et al., 1990). On the other hand, *Lactococcus lactis* produces EPS in the form of a

pentasaccharide containing glucose, galactose, galactose-1-phosphate and rhamnose (Nakajima et al., 1992). Although the number of EPS-forming LAB strains that have been characterized has been growing steadily, the important characteristic is their low level of EPS production, limiting practical use in industrial applications (de Vos, 1996).

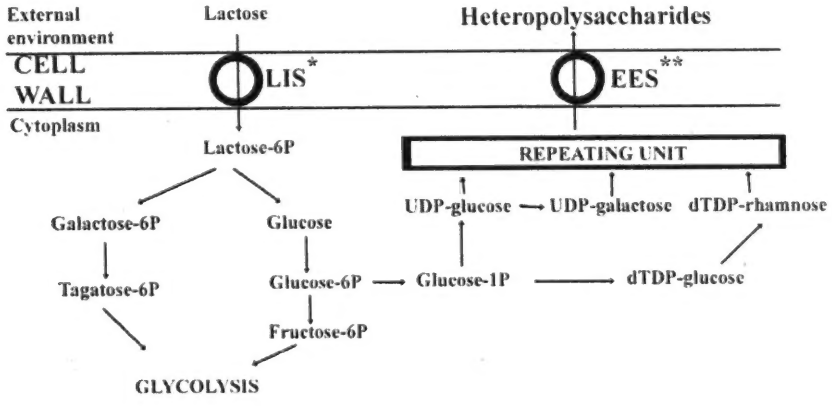

Figure 2.3. Highly schematic representation of EPS formation by *Lactococcus lactis*. Lactose is imported inside the cell by dedicated lactose import system (*LIS), cleaved by β-galactosidase and resulting monosaccharides are metabolized through the glycolysis or converted by several enzymatically controlled steps into EPS and excreted by EPS export system (EES) outside the cell. (NOTE: several steps omitted for simplification; adapted from de Vos, 1996)**

2.5.3. Lactobacillus delbrueckii ssp. *bulgaricus*

Lactobacillus delbrueckii ssp. *bulgaricus* is a Gram-positive, non-spore forming, non-motile rod which ranges from 0.5-0.8 μm in diameter and 2 to 9 μm in length. It grows well at 45°C, but is unable to grow at or below 15°C. Regarded as a facultative anaerobe, it also appears unaffected by the oxygen tensions, resulting in the aerobic growth comparable to that obtained in anaerobic environments (Bury et al., 1998). In the dairy processing industry, it can be a component of yogurt culture along with

Streptococcus thermophilus (Gilliland, 1985). This culture is also considered to be the highest β-galactosidase producing strain of all dairy cultures described in the literature (Friend et al., 1983; Shah and Jelen, 1990, 1991). As an obligate homofermentative bacterium, *Lactobacillus delbrueckii* ssp. *bulgaricus* ferments sugars to lactic acid via the EMP pathway, but is unable to utilize pentoses or gluconate (Hammes and Vogel, 1992). The galactose moiety of lactose is not utilized by *Lactobacillus delbrueckii* ssp. *bulgaricus*; similar to *Streptococcus thermophilus* (de Vos and Vaughan, 1994), galactose is excreted outside the cell by the secondary lactose-galactose antiport system with concomitant import of lactose (Figure 2-4, part A).

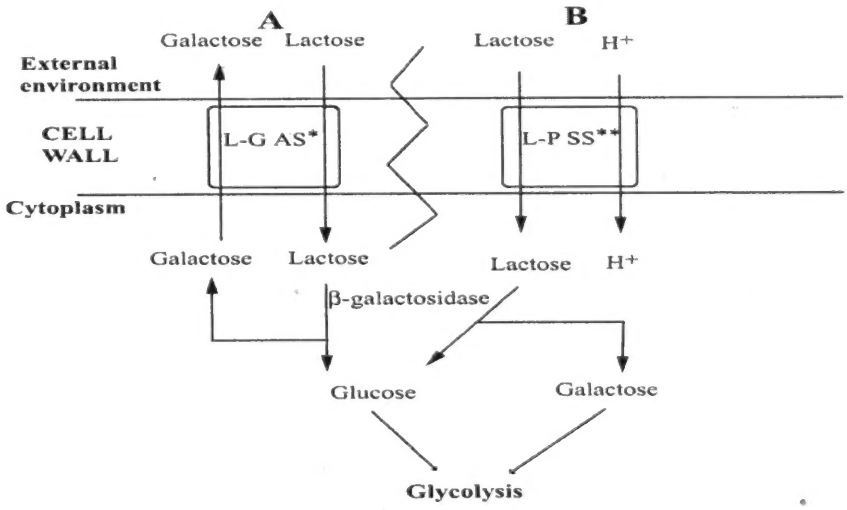

Figure 2-4: A) Lactose-galactose antiport (*L-G AS) or B) lactose-permease simport (L-P SS) system in Lactic Acid Bacteria (adapted from de Vos and Vaughan, 1994)**

The inability of *Lactobacillus delbrueckii* ssp. *bulgaricus* to metabolize galactose might be a reason for the high β-galactosidase production; to sustain rapid growth, it would have to hydrolyze twice as much lactose to produce the same amount of energy as

those microorganisms capable of utilizing galactose. The production of β-galactosidase is inducible (Leong-Morgenthaler et al., 1991). Hickey et al. (1986), measuring the β-galactosidase activity of different strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* cultivated in different media, reported a several fold increase in the β-galactosidase activity after the lactose addition. This β-gal activity was repressed after the addition of glucose or galactose.

The optimal pH for the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* in the lactose modified MRS broth (de Mann et al., 1960) is 5.8 (Venkatesh, 1998). Product inhibition was evident when the concentration of lactic acid exceeded 60 g/L, while lactose was inhibitory at concentrations over 80 g/L. *Lactobacillus delbrueckii* ssp. *bulgaricus* is unable to synthesize many of the essential compounds it requires, and depends on the availability of amino acids, vitamins, purines, pyramidines, and other factors in a culture medium for growth. The biosynthetic ability of lactobacilli is so limited that even humans have simpler vitamin requirements (Brock and Madigan, 1988). *Lactobacillus delbrueckii* ssp. *bulgaricus* will grow relatively slowly in whey or whey permeate, but the fermentation time can be substantially reduced by suitable supplementation of corn steep liquor (Cox and MacBean, 1977), molasses (Gupta and Gandhi, 1995; Chiarini et al., 1992), yeast extract (Gupta and Gandhi, 1995; Abd El Hafez et al., 1994; Parente and Zottola, 1991) or whey protein concentrates and isolates (Bury et al., 1998; Bury et al., 2000). It has been well established that *Lactobacillus delbrueckii* ssp. *bulgaricus* grows well in skim milk (Stefanitsi et al., 1995). However, the concentration of free amino acids and peptides in milk is very low, resulting in the

dependence of *Lactobacillus delbrueckii* ssp. *bulgaricus* on a proteolytic system allowing the degradation of milk proteins for growth (Juillard et al., 1995).

2.6. LAB β-galactosidases

The enzyme β-galactosidase (β-D-galactoside-galactohydrolase, EC 3.2.1.23) commonly referred to as β-gal or lactase, catalyzes the hydrolysis of β-D-galactosides and α-D-arabinosides. The β-D-galactosidase of *E. coli* has been the most studied, however the use of this enzyme in the food processing industry is prohibited due to the pathogenic character of *E. coli*. The industrially used β-gal is derived from yeasts and molds, but the identification and characterization of β-gal from different organisms is an ongoing process. Special attention has been focused on the potential of psychrotrophic and thermophilic LAB as β-gal sources due to their GRAS status (Mahoney, 1997; Coombs and Brenchley, 1999). The high β-gal activity of psychrotrophs at lower temperatures (about 5°C) or thermophilic cultures at elevated temperatures (over 50°C) effectively prevents microbial growth at even neutral pH (Mahoney, 1997). The most investigated LAB in regard to β-gal activity have been the strains of *Streptococcus thermophilus* (Rao and Dutta, 1981; Greenberg and Mahoney, 1982) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Itoh et al., 1980; Shah and Jelen, 1991). The structural organization of the chromosomal *lac*operon of these two organisms is similar and both were cloned in *E. coli* (de Vos and Vaughan, 1994). The DNA sequences of the *lac*Z gene, encoding for the β-galactosidase, were identical at the amino acid level except for an extra lysine residue at position 905 in the *Lactobacillus delbrueckii* ssp. *bulgaricus* derived gene. *Lactobacillus delbrueckii* ssp. *bulgaricus lac*Z gene codes for a protein monomer of 1,006 amino acids

with a calculated molecular weight of 113,915 daltons, slightly lower than the *E. coli* β-galactosidase monomer (Schmidt et al., 1989). While the β-gal from *E. coli* is an allosteric enzyme with a quaternary structure consisting of four monomeric units (Wallenfels and Weil, 1970), the quaternary structure of β-galactosidase from *Lactobacillus delbrueckii* ssp. *bulgaricus* has not yet been determined.

Lactobacillus delbrueckii ssp. *bulgaricus* β-galactosidase has an optimum activity temperature in the range of 50 to 55°C at neutral pH (Itoh et al., 1980; Shah and Jelen, 1991). The kinetic determinations showed that the lactose hydrolysis in a buffered lactose solution by this enzyme followed first order reaction kinetics with K_m and V_{max} values of 4.59mM and 10.1 μM min^{-1} mg^{-1}, respectively, with the higher affinity towards o-nitrophenyl-β-D-galactopyranoside (ONPG) (Itoh et al., 1980). The enzyme is slightly inhibited by the end products of hydrolysis as well as by sodium salts. At elevated temperatures over 50°C, it is more stable in milk than in a buffered solution. This stabilization effect is the result of the combined action of potassium, magnesium and manganese salts and other milk constituents, such as casein, whey proteins and lactose (Itoh et al., 1980; Greenberg et al., 1985; Kreft and Jelen, 2001).

2.6.1. Mechanism of lactose hydrolysis by β-galactosidase

Generally, the role of β-gal is to hydrolyze lactose to glucose and galactose. Additionally, β-gal converts lactose to other disaccharides, preferentially either allolactose (galactopyranosyl-1-6-β-D-glucopyranose; Huber et al., 1976) or higher oligosaccharides through various transferase reactions (Figure 2-5).

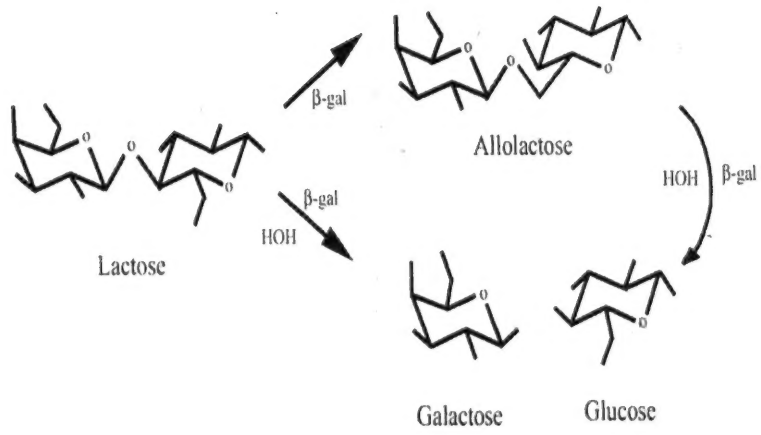

Figure 2-5: General scheme for the action of β-galactosidase on lactose. (adapted from Huber et al., 1976) The enzyme either hydrolyzes lactose (lower path) or catalyzes transferase reactions (upper path).

The enzyme is specific at the galactosyl position but is adept at hydrolyzing β-D-galactopyranosides with a wide variety of aglycons. This fact allows the use of simple, colorimetric assays based on substrates such as ONPG, which has chromogenic aglycons. The enzyme catalyses hydrolysis of its substrates in a double displacement reaction, involving an enzyme nucleophile, where the product preserves the same stereochemistry as the starting state (Sinnott, 1990). A proposed mechanism of β-gal action on lactose, schematically presented in Figure 2-6, involves the participation of a glutamic acid residue in the position 537 of the enzyme active center in nucleophilic catalysis of glycoside cleavage to give a covalent glycosyl-enzyme intermediate. Another glutamic acid residue in the position 461 contributes to reversible acid/base catalysis at the alkoxy leaving group/nucleophile. An enzyme-bound magnesium ion provides

electrophilic stabilization of a developing negative charge at the leaving group/alkyl alcohol nucleophile (Sinnott, 1990; Richard et al., 1996; Juers et al., 2001).

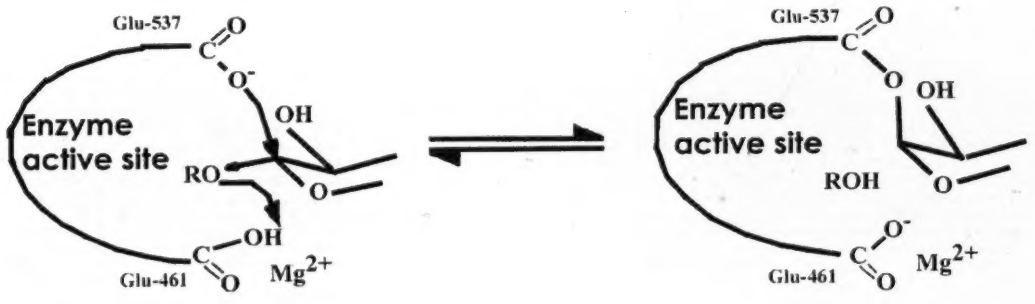

Figure 2.6: Hypothetical mechanism of the β-galactosidase action on lactose (RO$^-$ – acceptor sugar or water; adapted from Sinnot, 1990, and Juers et al., 2001)

If the nucleophilic compound is water, the final product will be galactose; however, when the nucleophilic compound is another sugar, the end result will be the formation of di-, tri-, and higher saccharides, collectively termed oligosaccharides (Mahoney, 1997).

2.6.2. Oligosaccharide formation

The amount and nature of oligosaccharides formed by transferase reactions depends primarily on the enzyme source, concentration and nature of the substrate and the reaction time. The concentration of formed oligosaccharides usually ranges from 1 to 45% (Zarate and López-Leiva, 1990). Depending on the source, β-gal is able to produce up to 20 different oligosaccharides (Toba et al., 1981; 1985). The β-gal from strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* has been characterized as having the highest transferase activity in comparison to other LAB species in several studies (Toba et al., 1981; Garman et al., 1996).

Galacto-oligosaccharides are hydrolyzed slowly, approximately 10 fold slower in comparison to lactose, by human small-intestinal β-gal, resulting in possible gastrointestinal discomfort (Burvall et al., 1980). In contrast, there has been a growing scientific interest in using galacto-oligosaccharides as a bifidogenic factor for facilitation of growth of bifidobacteria in lower gastrointestinal tract (Modler et al., 1990). The literature on this subject is extensive and the reader can consult some recent reviews for further discussions (Hawkins, 1993; Ruperez, 1998; Sako et al., 1999; Vandenplas, 2002).

2.7. Extraction of enzymes from microorganisms

The growing interest in the production and isolation of enzymes from microorganisms has been stimulated by the increasing use of the enzymes in industrial applications. Currently most of the enzymes, with major commercial importance, are produced extracellularly; however, many enzymes, retained within the cells, are also considered to be potentially industrially applicable, including the β-gal from LAB (Chisti and Moo-Young, 1986). The liberation of intracellular material requires either genetic modification of the cell, resulting in extracellular excretion of a desired metabolite, or spontaneous cell lysis or the cell disintegration by mechanical, physical, chemical or enzymatic means (Chisti and Moo-Young, 1986). The effectiveness of a cell disruption process depends on cell properties such as the physical strength of the bacterial cell wall (Sauer et al., 1988) and on the intracellular location of enzyme (Kuboi et al., 1995). This review briefly elaborates on the mechanical methods for the cell disruption currently employed in our laboratory; the reader can find in-depth information on all available cell

disruption methods in recently published reviews (Middelberg, 1995; Geciova et al., 2002a).

The mechanical methods involving either solid shear, i.e. bead milling, or liquid shear, i.e. high pressure homogenization, have been frequently used for the disruption of cells. Generally, the equipment used for large scale cell disruption has been modified from that used for particle size reduction or emulsion formation in other industries. The main disadvantages of these methods are associated with high capital investment and energy costs, enzyme inactivation by shear-associated denaturation and excessive heating due to energy dissipation (Chisti and Moo-Young, 1986).

2.7.1. Sonication

The treatment of microbial cultures in suspension with ultrasound (greater than 20 kHz) results in cellular inactivation and disruption. Ultrasonication utilises the rapid sinusoidal movement of a probe within the liquid, characterised by high frequency (18 kHz to 1 MHz), small displacements (less than about 50 μm) and moderate velocities (a few m s^{-1}) (Guerlava et al., 1998; Kumar and Pandit, 1999). Ultrasonication produces cavitation phenomena when acoustic power inputs are sufficiently high to allow the multiple production of microbubbles at nucleation sites in the fluid. The bubbles grow during the rarefying phase of the sound wave, then collapse during the compression phase. On collapse, a violent shock wave passes through the medium. The whole process of gas bubble nucleation, growth and collapse due to the action of intense sound waves is called cavitation. The collapse of the bubbles converts sonic energy into mechanical energy in the form of shock waves equivalent to several thousand atmospheres (300

MPa) pressure. This energy imparts motions to parts of cells that disintegrate when their kinetic energy content exceeds the wall strength (Wang et al., 1979). An additional factor that increases cell breakage is the microstreaming - very high velocity gradients causing shear stress which occur near radially vibrating bubbles of gas caused by the ultrasound (Chisti and Moo-Young, 1986). Generally, protein release is used as a measurement of the effectiveness of the ultrasonication, which follows a first order reaction:

$$\frac{dP}{dt} = -kP \qquad \text{Formula 2.1.}$$

where P stands for the protein concentration and coefficient k depends on the frequency and power input intensity (Save et al., 1997; Feliu et al., 1998; Kapucu et al., 2000). While the cell concentration has no effect on the amount of liberated proteins (Feliu et al., 1998; Bury et al., 2001), the ultrasonication efficiency is inversely correlated with the sample volume (Feliu et al., 1998)..

Although ultrasonication devices can be scaled up and operated continuously, they are not used on the industrial scale for the disruption of microorganisms. This could be attributed to the generation of excessive heat during the ultrasonication as well as high costs. Most of the acoustic energy is absorbed by the suspension and converted to heat, thus the efficient dissipation of heat is essential (Chisti and Moo-Young, 1986). Additionally, adiabatic compression of the medium by acoustic radiation and high deceleration result in high localized temperatures (Wang et al., 1979). The enzyme inactivation may also be caused by ionization and subsequent free radical formation, in

addition to the detrimental effect of excessive heat, which denatures the enzyme (Wang et al., 1979).

2.7.2. Bead milling

The bead mills, originally developed for the pigment industry, provide grinding and dispersion by interparticle collision and solid shear. They consist of either a vertical or a horizontal grinding chamber containing rotating discs or impellers mounted on a motor driven shaft (Figure 2-7). These impeller devices accelerate the glass or plastic beads to supply a grinding action. An efficient cooling system, usually in the form of a cooling jacket, is required to dissipate the generated heat.

The use of the bead mill for the microbial disruption has been tested on yeasts (Schütte et al., 1983; Mao and Moo-Young, 1990) and both gram positive and gram negative bacteria (Schütte et al., 1983; Bury et al., 2001; Geciova et al., 2002b). The process is influenced by a wide range of parameters, including bead diameter, density and loading, cell concentration in feed, flow rate of feed, agitator speed and configuration, geometry of the grinding chamber and temperature. In general, the following equation can be used to estimate the efficiency of the process using the release of proteins from microbial cells (Kula and Schütte, 1987):

$$\ln\left(\frac{P_m}{P_m - P}\right) = -k \cdot t$$

Formula 2.2.

in which k is a first order rate constant, P_m and P are the maximum protein concentration released by bead milling and protein concentration at time t, respectively.

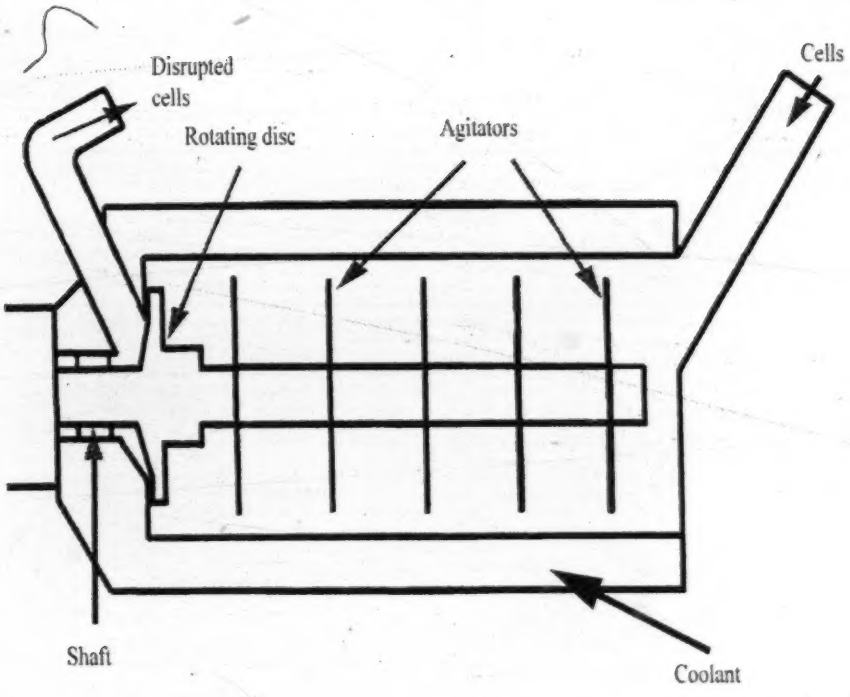

Figure 2-7: Schematic illustration of a bead mill
(adapted from the literature of a manufacturer; Glen Mills Inc., Clifton, NJ, USA)

In general, smaller bead sizes are more effective. Typically for yeast cells, beads of 0.25 to 0.75 mm diameter are preferred. Using larger beads, enzymes located in the periplasmic space can be preferentially released whereas smaller beads are required for the release of cytoplasmic enzymes (Schütte et al., 1983). Disintegration of bacteria is hampered by their relatively small size, requiring bead size reduction for the efficient disruption. On the other hand, the use of smaller beads is limited due to their tendency to float (Schütte et al., 1983). The cell concentration has little or no effect on the effectiveness of the process (Schütte et al., 1983; Bury et al., 2001), while the bead load is directly correlated not only with the degree of the cell disintegration but also power

consumption. A range of 80 to 85% bead load is considered optimal (Kula and Schütte, 1987).

2.7.3. High pressure homogenization

High-pressure homogenization is one of the most widely known methods for the large scale cell disruption. A positive displacement piston pump is used to draw the cell suspension through a check valve into the pump cylinder. On return of the piston, the suspension is forced through the adjustable annular gap of a discharge valve and impinges on an impact ring. The discharge pressure, regulated by a valve rod, controls the position of the valve in relation to the valve seat (Figure 2-8).

The impingement and impact are the main causes for the cell disruption (Moore et al., 1990), although the hydrodynamic cavitation has also been reported as a contributing factor (Shirgaonkar et al., 1998; Wuytack et al., 2002). The homogenizer has been principally used in the dairy industry for the fat globule size reduction, employing a two-stage pressure up to 15 MPa. However, this pressure is rather ineffective for the cell disruption and higher pressures, in the range 55 to 300 MPa, are employed for this task (Wuytack et al., 2002).

Homogenization has been successfully used for the disruption of yeasts (Shirgaonkar et al., 1998) and bacteria (Bury et al., 2001; Geciova et al., 2002b; Wuytack et al., 2002). The effectiveness of disruption is generally found to be pressure and temperature dependent (Chisti and Moo-Young, 1986), while the cell concentration has no apparent effect (Bury et al., 2001). The degree of disruption is further enhanced by increasing the number of passes (Bury et al., 2001); however, concomitant heat

generation may result in the enzyme deactivation (Geciova et al., 2002b). The equation 2.3 has been successfully used as a starting point in describing the efficiency of the high pressure homogenization (Kumar and Pandit, 1999):

$$\ln\left(\frac{P_m}{P_m - P}\right) = k \cdot N \cdot p^a \qquad \text{Formula 2.3}$$

where P_m is the maximum amount of protein available for release, P is the amount of protein released at time t, k is a rate constant, N is the number of passes, p is the operating pressure, and a is a constant, which depends on the microorganism and conditions of its growth.

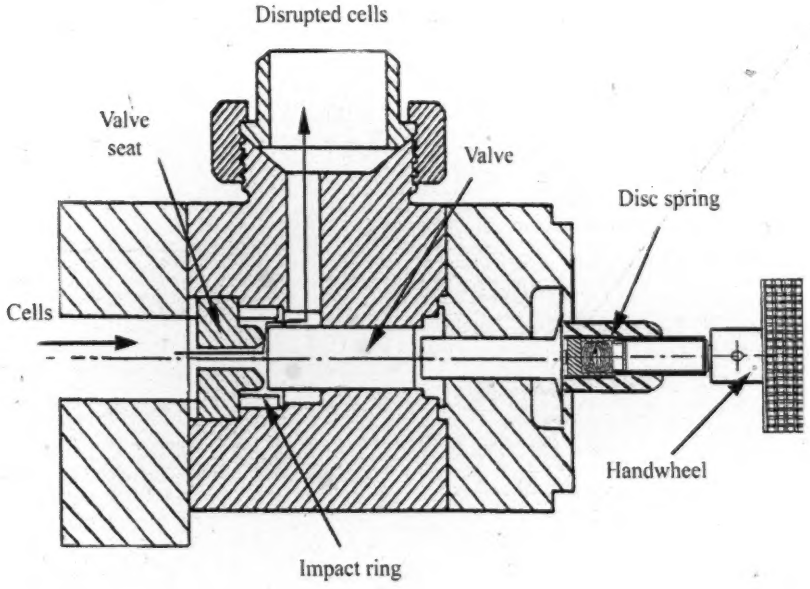

Figure 2-8: High-pressure homogenizer discharge valve unit (adapted from the documentation of a manufacturer, APV Americas – Homogenizer, Wilmington, MA, USA)

Distinctly different pressures are required to disrupt different microorganisms (Wuytack et al., 2002). Gram-negative bacteria are easier to disintegrate than gram-positive bacteria and filamentous fungi, which in turn are easier to disrupt than unicellular yeasts. Such a pattern is generally attributed to the cell wall composition, size, shape and growth phase of the microorganism (Chisti and Moo-Young, 1986).

2.7.4. Microfluidization

The Microfluidizer® Processor (Microfluidics Co., Inc., Newton, MA, USA) is an ultra high-pressure mixer, homogenizer and particle/droplet size reducer used in many applications in food, pharmaceutical, coating, and cosmetic industries. Its principle of operation is based on pumping a liquid product at constant high pressure (up to 275 MPa) with subsequent splitting of the main stream into two smaller ones, which are then forced to collide against each other within an interaction chamber. Forces applied to the product are intense: shear forces as the product travels along the walls of microchannels at high velocity, impact against the walls of the interaction chamber, impact of the two streams colliding, and cavitation caused by bubbles forming and collapsing as the stream passes through various zones of pressure differentiation within the interaction chamber (Barnadas-Rodriguez and Sabes, 2001). The disrupting unit is schematically presented in Figure 2-9.

The effectiveness of the disruption is a function of pressure, number of passes and the cell concentration in suspension (Middelberg, 1995). The use of the Microfluidizer® may be favorable for the cell disintegration in comparison to the high-pressure homogenizer, since the efficient cell disruption can be achieved using one-pass through

the chamber. In a recent study, over 90% disruption was achieved for *Streptococcus thermophilus* 143 (Geciova et al., 2002b) The heat generation in the disrupting chamber is easily dissipated by efficient cooling, i.e. submerging the chamber in an ice-water bath, preventing the enzyme inactivation (Geciova et al., 2002b).

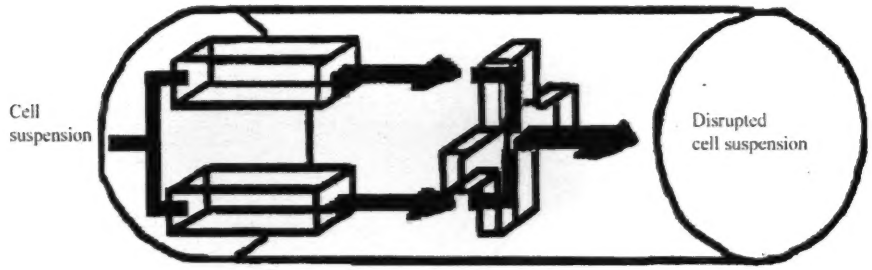

Figure 2-9: Conceptualized stream flow in a Microfluidizer® disrupting chamber (adapted from the manufacturer's documentation; Microfluidics, Newton, MA, USA)

2.8. Stability of enzymes and enzyme containing materials

An important factor in production and application of bioactive materials is that many of them are very unstable, i.e. many enzymes lose some or all of their catalytic activity during either recovery or application. Enzyme stability is usually defined as the ability to retain catalytic activity (Weijers and Van't Riet, 1992). Destabilizing pathways for enzymes can be distinguished into two distinct classes, involving chemical instability and physical instability. Chemical instability can be defined as any enzyme modification that involves bond formation or cleavage, resulting in a new compound. These modifications can include hydrolysis of the peptide bonds as well as deamidation of asparagine and glutamine side chains. Oxidation of cysteine can lead to disulfide bond formation, while oxidation of methionine and other amino acids may inactivate or alter

enzyme activity (Manning et al., 1989). The physical instability involves changes in the higher order protein structure. These include denaturation, adsorption to surfaces, aggregation and precipitation (Kendrick et al., 1998).

Denaturation refers to an alteration of a global fold of a protein molecule, through a disruption of the tertiary and frequently secondary structure (Brems et al., 1985; Baum et al., 1989). Although inactivation may be caused by several mechanisms (see above), denaturation remains the most widely studied aspect of the protein instability (Manning et al., 1989). Although possibly caused by wide range of conditions such as high temperature, extreme pH, or addition of a denaturant, protein inactivation through denaturation can be described as a two-step process (Weijers and Van't Riet, 1992):

$$N \rightleftharpoons D \longrightarrow I$$

Formula 2.4.

The native state (N) partially unfolds to a reversibly denatured state (D). Further unfolding results in a secondary step, which leads to an irreversibly denatured state (I). The irreversibly inactivated enzyme is incapable of reactivating spontaneously upon removal of an applied stress, i.e. excessive heat, or returning to physiological conditions. Several interactions, such as electrostatic, hydrogen, hydrophobic, dipolar and disulfide bonds, contribute to the conformational stability of the enzyme native state (Whitaker, 1994). The total free energy of protein stabilization is low (40 to 120 kJ mol^{-1}), resulting in a marginal thermodynamic stability of the native form (Weijers and Van't Riet, 1992). Thus minor environmental changes may result in a negative free energy of stabilization and subsequent denaturation.

In general, the aim of stabilization is the suppression of one or more of the inactivation processes. Apparently, the unfolding of the protein globule is the rate-limiting step during protein denaturation, thus the inhibition or suppression of unfolding presents a general approach for enzyme stabilization (Bhuyan, 2002; Castronuovo et al., 2002). Also, the prevention of the secondary irreversible reactions by application of different stabilizers or manipulation of the processing conditions contributes to improved enzyme stability (Weijers and Van't Riet, 1992).

2.8.1. Enzyme stabilization in solutions

The addition of various stabilizing solutes to enzyme solutions has been a common way of protecting them during preparation and storage. A wide variety of solutes, such as sugars, polyols, amino acids and salts, have been effective in preventing or minimizing the enzyme denaturation upon imposed stress, i.e. heat, in aqueous systems (Arakawa and Timasheff, 1982; 1983; 1984a; 1985). Although enzyme stabilizers come from different classes, the protective mechanism is universal and can be described by preferential hydration of proteins (Timasheff, 2002). The presence of stabilizing solutes in a protein solution results in increased chemical potentials of both the protein and the additive creating a thermodynamically unfavorable situation. Such an entropically unfavorable state leads to preferential exclusion of solutes in the vicinity of proteins and subsequent hydration and stabilization (Xie and Timasheff, 1997). The native structure of monomers and the polymerized form of oligomeric proteins are stabilized because denaturation and dissociation, respectively, would lead to a greater contact surface between the protein and the solvent, thus increasing this thermodynamically unfavorable

effect (Crowe et al., 1998). In contrast, solutes that preferentially bind to proteins cause, at certain solute concentrations, the protein unfolding and denaturation (Prakash et al., 1981; Arakawa and Timasheff, 1984b). Additional stress, such as high temperature or extreme pH, decreases the free energy difference between the native and denatured states and, thus, decrease the solute concentration needed to induce denaturation (Arakawa et al., 1990).

2.8.2. Enzyme stabilization by drying

Proteins and enzymes for commercial use are often dried in order to extend their shelf life (De Paz et al., 2002). However, the biological functions of proteins depend on their three-dimensional structure, which is determined largely by water. Water removal can lead to loss of native structure, resulting in loss of biological function upon rehydration (Prestrelski et al., 1993; Carpenter et al., 1994). Maintaining native protein structure during dehydration, and consequently maintaining the capacity to recover the biological function of the protein upon rehydration, is an important consideration in many areas of biochemical research. It was shown that anhydrobiotic organisms have the ability to preserve essential proteins in the desiccated state, as biological functions of the organism resume after rehydration prior to the synthesis of new proteins (Arakawa et al., 2001). Anhydrobiotes preserve the biological activity of their constituent proteins by accumulating disaccharides in their tissues during drying (Crowe et al., 1998). This strategy has been applied to the stabilization of purified proteins for which disaccharides have been found to inhibit protein unfolding during freeze– drying. Although observations on protein stabilization by disaccharides are largely empirical, the

mechanism by which carbohydrates protect dried proteins has been debated in the literature (Arakawa et al., 1990).

The usual method for the preparation of dried enzyme formulations is freeze drying (Ford and Dawson, 1993; Gubern et al., 1996; Sundari. and Adholeya, 2000a,b). The principal advantage of this dehydration process is that water removal is accomplished without exposing the product to excessively high temperatures. In general, the process involves initial freezing followed by application of heat to the product surfaces, which results in ice sublimation (Heldman and Singh, 1981). However, high maintenance cost and low capacity limit the applicability of this method for processing large volumes on an industrial scale. As an alternative, spray drying has been employed for the stabilization of various heat-sensitive biological materials including enzymes, foods (milk and egg), and pharmaceutical products such as antibiotics (Rasolomanana et al., 1984; Labrude et al., 1989; Mumenthaler et al., 1994). Despite the relatively high temperatures involved in the application of a hot air stream as a drying medium, the residence time of droplets in a spray drier is short (3 to 10 s) and the product particles may never be at a temperature higher than the wet bulb temperature of the drying air (Meerdink, 1994). Additionally, the low water content of the product leaving the drying chamber allows the labile compound to withstand the detrimental effect of heat (Etzel et al., 1996).

Nevertheless, the physical stress incurred on proteins in the drying process may cause protein degradation and, in the case of enzymes, irreversible loss of activity. Carpenter et al. (1987) and Carpenter and Crowe (1988) showed that the complete catalytic activity loss of phosphofructokinase, a tetrameric enzyme, upon dehydration

was due to dissociation into constitutive monomers. Two effects responsible for the loss of β-gal activity during spray drying were suggested - thermal degradation and surface denaturation in addition to protein conformational changes, affecting the enzyme kinetic properties (Luyben et al., 1982; Branchu et al., 1999). While a variety of solutes stabilized the protein conformation in aqueous systems due to solute preferential exclusion (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982), only sugars and sugar derivatives (Carpenter et al., 1987; Carpenter et al.; 1988; Arakawa et al., 1990; Yamamoto and Sano, 1992;Ramos et al., 1997 Branchu et al., 1999; Belghith et al., 2001) and salts (Werner et al., 1993) seem to be effective in preventing enzyme denaturation during dehydration by spray- or freeze drying.

Two mechanisms for stabilizing enzyme activity have been proposed depending on the kind of stress imposed on proteins. First, the water replacement theory states that protective solutes bind to the protein being dried, serving as a water substitute when the hydration shell of the protein is removed (Carpenter and Crowe, 1988). Second, the glassy state theory asserts that the preservation of enzyme activity is caused by physical entrapment of protein in an amorphous stabilizing compound matrix, subsequently preventing protein conformational changes (Izutsu et al., 1994). Lactose has been recognized as a protein stabilizing solute, mainly due to its readily attainable amorphous form (Yamamoto and Sano, 1992; Suzuki et al., 1997; Millqvist-Fureby et al., 1999a,b). The protein preservation was further enhanced if surfactants along with sugars were added to prevent the protein accumulation on the surface of droplets during spray drying (Fäldt and Bergenståhl, 1994; Millqvist-Fureby et al., 1999a).

2.9. Applications of lactose hydrolysis

Main products of lactose hydrolysis, glucose and galactose, have some advantages in comparison to unhydrolyzed lactose. They are sweeter than conventional products, which allows a reduction in the amount of sugar added to products such as ice cream or flavored milk formulated with hydrolyzed lactose milk, whey, or whey permeate (Arndt and Wehling, 1989).

The market for lactose hydrolyzed milk has been growing steadily, at a rate of approx. 20% per year in the US, mainly stimulated by increased awareness of the lactose intolerance problem and changing demographics (Mahoney, 1997). A market of 50 million lactose maldigesters exists in North America alone (Sloan, 1999). The information of lactose hydrolyzed products on the market is abundant and following examples were found on the internet, mainly on the web site of producers. Lactose-hydrolyzed or low-lactose milk is readily available at retail stores under brand names such as "Dairy Ease", a 70% lactose reduced milk (Dairy Ease, Blistex Inc., Oakbrook, IL), or "Lactaid", 70 or 100% lactose reduced milk (Lactaid, McNeal Consumer Products, Fort Washington, PA). Gelda Foods (Gelda Scientific, Mississauga, ON) recently introduced a lactose free ice cream, sold under the "Lacteeze" trade name.

A novel low-calorie and natural lactose-based bulk sweetener, with only 5 kJ g^{-1} and almost the same sweetening power as sucrose, has been developed by Arla Food Ingredients of Denmark. The sweetener, called Gaio®-tagatose, offers a variety of functionality and health benefits for use in a range of foods and beverages. The production of this sweetener is accomplished by a two-step process. In the first step, lactose is hydrolyzed by β-galactosidase and the resulting galactose, which is produced,

is converted through isomerization to tagatose. The ingredient has obtained FDA-notified GRAS status in the USA. Approval procedures are also pending in Japan, Europe, Australia, and New Zealand. Recently, the UK based company, Galactogen Products Limited introduced a range of sport drink products under the "G-Push Sport" brand name. The basic drink formulation contains galactose obtained through lactose hydrolysis as a sole carbohydrate source.

In addition, other products have been introduced, based on lactose hydrolysis by β-galactosidase. A process developed in 2000 by the Battelle company (Richland, WA) introduced the production of high value polyol products from cheese whey. The process involves three catalytic steps to break down and hydrogenate the lactose to produce polyol chemicals. In the first step, lactose is hydrolyzed by an immobilized β-galactosidase. During the remaining steps, the mixture of glucose and galactose is converted into a mixture of polyols, primarily propylene glycol, ethylene glycol, and glycerol.

Although extensive research has been conducted on the production of sweeteners from hydrolyzed whey (Holsinger and Kligerman, 1991), the low cost of commercially used sweeteners, such as corn syrup and high fructose corn syrup, imposes an economical constraint on a small scale production of lactose hydrolyzed sweeteners. Development of an economically feasible process for lactose hydrolysis would not only result in production of more economically competitive sweeteners, but also expand the possibilities for utilization of lactose-containing products. It would also likely lead to other innovative applications such as those dealt with in this work.

2.8. References

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Chapter 3*

Production of β-Galactosidase for Lactose Hydrolysis in Milk and Dairy Products Using Thermophilic Lactic Acid Bacteria

3.1. Introduction

The enzyme β-D-galactoside galactohydrolase (β–galactosidase, E.C. 3.2.1.23, trivially lactase) hydrolyzes lactose, the milk sugar, into two moieties - glucose and galactose. The decline of lactase activity in human small intestine, caused by the rate of lactase gene transcription (Lee and Krasinski, 1998), is prevalent in more than half of the world's population (Rings et al., 1994). The development of lactose hydrolyzed products presents one of the possible approaches to diminish the lactose maldigestion problem. A technically and economically feasible process would also open new possibilities for the utilization of whey and whey permeate to obtain value-added products while eliminating some potential technological problems such as sandiness in ice cream (Zadow, 1993) or whey cheese (Patocka and Jelen, 1988).

The choice of lactose hydrolysis process technology depends primarily on the nature of the substrate, the enzyme characteristics and the economics encompassing the production, storage and marketing of the product (Mahoney, 1997). Technically feasible processes include the direct addition of soluble enzyme; recycling of the soluble enzyme by membrane processes; or use of immobilized enzymes (Jelen, 1993). An inexpensive batch processing using in-house production of crude enzyme preparations could make manufacturing of lactose hydrolyzed products feasible even for smaller dairy plants.

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While β-galactosidase has been found in numerous biological systems, microorganisms such as yeasts, molds and bacteria still remain the only commercially exploited sources (Agrawal et al., 1989). The lactase characteristics are strongly origin related, thus different applications might require enzymes from different sources. More recently, thermophilic bacteria have become an object of interest for the commercial production of lactase (Petzelbauer et al., 1999). Among these bacteria, special attention has been paid to lactic acid bacteria (LAB) because of their GRAS status (Stiles and Holzapfel, 1997). The lactase of a yogurt mixed culture, consisting of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, has already been characterized (Itoh et al., 1980; Greenberg and Mahoney, 1982). Both enzymes showed high activity and stability at temperatures above 50°C. Such conditions enhance the rate of lactose hydrolysis as well as prevent the growth of undesirable microorganisms. However, for the production of the β-galactosidase, the fastidious nature of LAB requires numerous growth factors such as minerals and vitamins in addition to carbohydrate and nitrogen sources to be present in a growth medium (Stiles and Holzapfel, 1997). The use of an expensive complex medium would greatly increase the production costs. Therefore, the formulation of a suitable medium has been sought using an inexpensive and readily available components such as whey and whey permeate, without or with supplementation (Bury et al., 2000; Bury et al., 1998; Gupta and Gandhi, 1995; Chiarini et al., 1992; Parente and Zottola, 1991; Cox and MacBean, 1977). However, most of the work conducted so far has been focused on an enhancement of lactic acid or biomass production and very little on the maximization of β-galactosidase production by lactic acid bacteria.

The lactase from thermophilic LAB is an intracellular enzyme and its application from an in-house grown culture will have to include a removal of the culture from a medium and enzyme release by disruption of the microbial cells. While the enzyme liberation costs could be minimized (Shah and Jelen, 1991), the culture separation would leave a great deal of medium to be reprocessed (Bury and Jelen, 2000). Unlike the medium used for the dairy starter culture production, which may be directly added into a product along with the culture as in the case of cheese making, the spent medium used for the production of the β-galactosidase would be regarded as a waste. Alternatively, it could be converted into valued products diminishing the problem of its disposal.

Lactic acid bacteria, isolated from milk products, require from 4 up to 14 amino acids depending on the strain (Chopin, 1993). However, the amount of free amino acids and peptides in milk is very low. Therefore, lactic acid bacteria depend on a proteolytic system allowing degradation of milk proteins for the growth (Juillard et al., 1995). Casein, comprising the major part of milk proteins, contains all amino acids necessary for the growth of lactic acid bacteria in milk to high cell density, yet only a minor fraction, less than 1% of the total casein content, is actually needed (Kunji et al., 1996). It has been well established that *Lactobacillus bulgaricus* grows well in skim milk (Gilbert et al., 1996). Therefore, skim milk could present a suitable reprocessable medium for the cultivation of the potential β-galactosidase source *Lb. bulgaricus* 11842 containing all the necessary nutrients in the form of lactose and protein source.

The aim of this research was to compare the suitability of several dairy and dairy product based media in order to obtain the maximum growth and β-galactosidase activity production by the *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 cultures and

to propose a possible technological process for an in-house lactase production and reprocessing of the growth medium.

3.2. Materials and methods

3.2.1. Microorganism and Media

Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842 was obtained from University of Alberta, Department of Agricultural, Food and Nutritional Science. This dairy culture was chosen for its well-known high lactase producing activity (Shah and Jelen, 1991). The culture was propagated every second day in sterile MRS broth (Difco, Becton-Dickenson, MA) enriched with 1% (w/v) α-lactose monohydrate (Fisher Scientific Co., New Jersey). To prepare the culture for fermentation, about 0.5 mL of refrigerated culture was added to 5 mL of the lactose enriched MRS broth. After incubation at 43°C for 8 to 12 hours, 1 mL of active culture was used to inoculate 150 mL of sterile broth containing 3% (w/v) reconstituted whey powder and 1.3% (w/v) MRS (Difco) powder (WMRS broth). This culture was incubated at 43±1°C and 150±25 RPM in a controlled environmental incubator shaker (New Brunswick Co., USA) for 10 - 12 hours.

The whey (W) and whey permeate (WP) based broths used for the fermentations were prepared by dissolving 6 g spray dried whey (Dairy World Foods, Vancouver, BC) or whey permeate powder (Maple leaf Foods International, Toronto, ON) per 100 mL distilled water, adding one or more of the tested adjuncts. The adjuncts consisted of powdered yeast extract (YE) (Fermtech, BDH Inc, Toronto), two whey protein concentrates (WPC1 and WPC2) and a whey protein isolate (WPI) supplied, in random

order, by Promil, Novy Bydžov, Czech Republic; DMV USA, La Crosse, WI (product R-80); and Land-O-Lakes Inc., St. Paul, MN (product #27361). Commercial skim milk (Dairy World Foods, Vancouver, BC) and 1.2% (w/v) MRS enriched whey or whey permeate broths were used as protein rich media. Whey or whey permeate unsupplemented broths served as controls. The random codings and formulations of all media are presented in Table 3-1. Two 2000 mL flasks containing 750 mL of broth were autoclaved at 121°C for 15 minutes and aseptically transferred to a sterile 2 L fermentor (New Brunswick Co., USA).

Table 3-1: Coding and formulation of whey and whey permeate media used in β-galactosidase-producing fermentations

Code	Formulation
W (control)	Dried whey powder (6% aqueous solution)
WYE	W + 0.2% yeast extract
WMRS	W + 1.2% MRS
WWPC1	W + 0.2% yeast extract + 1% WPC1
WWPC2	W + 0.2% yeast extract + 1% WPC2
WWPI	W + 0.2% yeast extract + 1% WPI
WP (control)	Dried whey permeate powder (6% aqueous solution)
WPYE	WP + 0.2% yeast extract
WPMRS	WP + 1.2% MRS
WPWPC1	WP + 0.2% yeast extract + 1% WPC1
WPWPC2	WP + 0.2% yeast extract + 1% WPC2
WPWPI	WP + 0.2% yeast extract + 1% WPI

3.2.2. Fermentation

The 1.5 L batch of a fermentation broth was inoculated with approximately 50 mL of the culture grown in the WMRS broth. The temperature was controlled by placing the fermentor in a water bath at 43 ± 0.2°C. The pH was maintained at pH 5.6 ± 0.1 by the

automatic addition of 2N NaOH via a peristaltic pump (Watson-Marlow, USA) controlled by a pH controller (Omega Engineering Inc., USA). All fermentations were performed aerobically in a batch mode. During fermentations, the rate of acidification was inferred from the amount of 2N NaOH added. Viable counts were enumerated as colony forming units (CFU) mL^{-1} by surface plating dilutions on the MRS medium (55 g L^{-1}, Difco) with agar (15 g L^{-1}, Difco) and incubating under aerobic conditions at 43°C for 48 hours. All trials were replicated four times.

3.2.3. Chemical analysis of whey protein products

The three whey protein products (WPP) were analyzed for their contents of moisture, ash, protein, lactose and selected minerals, including calcium, magnesium, sodium, potassium, and phosphorus. The moisture content was determined by oven drying 5 mL samples at 110°C for 4 hours. For ash and mineral determination, 5 mL of a sample was dried at 125°C for 2 hours, then ashed at 550°C for 4 hours, and further treated according to the method of Gaines and West (1990). Calcium, magnesium, sodium and potassium were determined by atomic absorption spectrophotometry, using a Perkin-Elmer 4000 AA spectrophotometer (Perkin-Elmer, Norwalk, CT). Phosphorus was measured by the phosphomolybdate colorimetric method (Pulliainen, and Wallin, 1996). Total and non-protein nitrogen were determined using the official AOAC method (1995). The non-protein nitrogen was obtained by the precipitation with 12% TCA. The protein content was obtained as the nitrogen content multiplied by 6.38 and expressed as the total protein and TCA-soluble nitrogen. Lactose was determined by high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) equipped with a refractometer.

Separation took place in an Aminex HPX-87H, 300 x 7.8 mm ion exclusion column (Biorad, Hercules, CA) equipped with a guard column (Phenomenex, Torrence, CA) at 35°C with 5 mM H_2SO_4 as mobile phase at flow rate of 0.5 mL min^{-1}. The buffering capacities of investigated broths were assessed according to Hickey et al. (1983), using 1.4 N solution of lactic acid and heat sterilization instead of sodium azide as a preservation method. The buffering capacity was expressed as the amount of lactic acid in µmol required to decrease the pH of 1 mL of media by 1 unit.

3.2.4. Determination of β-galactosidase activity

For determination of the β-galactosidase activity, approximately 5 mL of a culture was sonicated in a 15 mL polypropylene centrifuge tube using the intermediate tip of a Sonic 300 dismembrator (Artek Systems Corporation, Farmingdale, USA) at 60% intensity. The sample was cooled using an ice water bath to prevent activity loss during sonication (Shah and Jelen, 1991). The β-galactosidase activity of the culture was assessed in the sonicated suspension using o-nitrophenyl-ß-D-galactopyranoside (ONPG) as the substrate at 37°C and pH 7 maintained in phosphate buffer (Shah and Jelen, 1991). The concentration of o-nitrophenol (ONP) released was determined from the absorbance at 420 nm, using the standard calibration curve (Spectronic 21, Bausch and Lamb, USA). The enzymatic activity was expressed as units of β-galactosidase activity per mL of undiluted culture. A unit of activity was defined as the amount of the enzyme, which liberated 1 µmol of ONP from ONPG per minute per mL of media under experimental conditions described above. All solutions were cooled in ice water. Experiments and all analyses were replicated and duplicated at least once. Unless indicated otherwise, results

are expressed as average ± standard deviation (SD) using all available data. Statistical significance of differences ($p < 0.05$) was determined by ANOVA and t-test where appropriate. The correlational analysis was conducted by using the Excel Analysis ToolPak (Microsoft Office 2000).

3.2.5. Processing of the "spent" skim milk

The fermentation of skim milk was carried out as described above and terminated after 10 hours. The medium was centrifuged (Beckman model J2-21, Beckman Coulter Inc, Fullerton, CA) at 4°C and *4500 x g* for 10 min for the culture separation and collection. The supernatant, referred to as spent skim milk (SSM), was collected and processed using high pressure ultrafiltration (HPUF), simulating a nanofiltration effect (Vasiljevic and Jelen, 2000a), to a volume concentration ration (VCR) of 2. The SSM was concentrated on a pilot-scale RO-UF apparatus DDS LAB-20 (De Danske Sukkerfabrikker, Nakskov, Denmark), containing only one sandwich (a support plate with a membrane on each side) with an effective filtration area of 0.036 m^2. The complete description of the experimental set-up can be found in Tarnawski and Jelen (1985). The GR60PP (polysulfone-polypropylene) UF membrane, with the nominal molecular weight cut-off of 25 kDa was supplied by Union Filtration (Nakskov, Denmark). A 4 L batch of SSM was circulated from an insulated feed tank of 20 L capacity through the module with a high pressure piston pump (model 16.50, Rannie, Copenhagen) at 10°C (±1), at 30 bars and a flow rate of 6 L min^{-1}. A batch of commercial fluid skim milk (SM) (Dairy World Foods, Vancouver, BC) was ultrafiltered in the same manner. After HPUF, SSM retentate (SSMR) and skim milk permeate (SMP) were

recombined in a 1:1 volumetric ratio. Samples of SSM and SM, SSMR and SMP, and recombined product (RP), taken before and after HPUF and after recombination, were frozen and stored for chemical analysis of total solids, ash, calcium, potassium and sodium as described above. A portion of RP was mixed with 5% (w/w) commercial sour cream (Dairy World Foods, Vancouver, BC) and cultivated aerobically in a water bath at 30°C for 12 hours. Another portion was combined with 5% (w/w) commercial stirred plain yogurt (Western Family Foods, Vancouver, BC) and fermented aerobically in a gravity convection incubator (Precision Scientific Group, Chicago, IL) at 43°C for 4 hours. The remaining portion of RP was inoculated with approximately 2% of *Lb. bulgaricus* 11842 and cultivated similarly to the yogurt resembling product to prepare a product resembling a lactic drink. A cursory taste evaluation of RP, RP sour cream and RP lactic drink was conducted by three experienced dairy product evaluators.

3.2.6. Determination of kinetic parameters

The logistic equation of Moraine and Rogovin (1966) was used to model the culture growth kinetics:

$$\frac{dX}{dt} = \frac{\mu X}{(1+\mu X)}$$

Formula 3.1

where X is the cell concentration (CFU mL^{-1}) and μ, the specific growth rate (h^{-1}). The integrated form of this equation, with X_0 as cell concentration (CFU mL^{-1}) at the beginning of fermentation (t = 0), and X_m, the maximum cell concentration (CFU mL^{-1}) obtained during fermentation, gives a sigmoidal curve empirically representing both exponential and stationary phases:

$$X(t) = \frac{X_0 e^{\mu t}}{1 - \frac{X_0}{X_m}(1 - e^{\mu t})}$$

Formula 3.2.

The Luedeking and Piret equation (Luedeking and Piret, 1959) was used to describe the kinetics of lactic acid production during fermentations:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$$

Formula 3.3.

where P is the instantaneous lactic acid concentration (mg mL^{-1}) and α and β are, respectively, the growth and non-growth (maintenance) associated lactic acid production constants. Both coefficients are dependent upon the strain, growth medium, and fermentation conditions. The maintenance constant can be obtained from stationary growth phase (dX/dt = 0), while the procedure described by Luedeking and Piret (1959) and Weiss and Ollis (1980) is used to linearize the experimental data in the exponential phase for the determination of the growth associated constant. The lactic acid production associated with the growth was estimated from:

$$P_\alpha = \alpha \frac{X_0 (1 - \frac{X_0}{X_m})(e^{\mu t} - 1)}{1 - \frac{X_0}{X_m}(1 - e^{\mu t})}$$

Formula 3.4.

while the non-growth associated lactic acid production was calculated as:

$$P_\beta = \beta \frac{X_m}{\mu} \ln\left[1 - \frac{X_0}{X_m}(1 - e^{\mu t})\right]$$

Formula 3.5.

Parameters μ, α, β, P_α and P_β were determined for each fermentation run and average values for each medium were calculated.

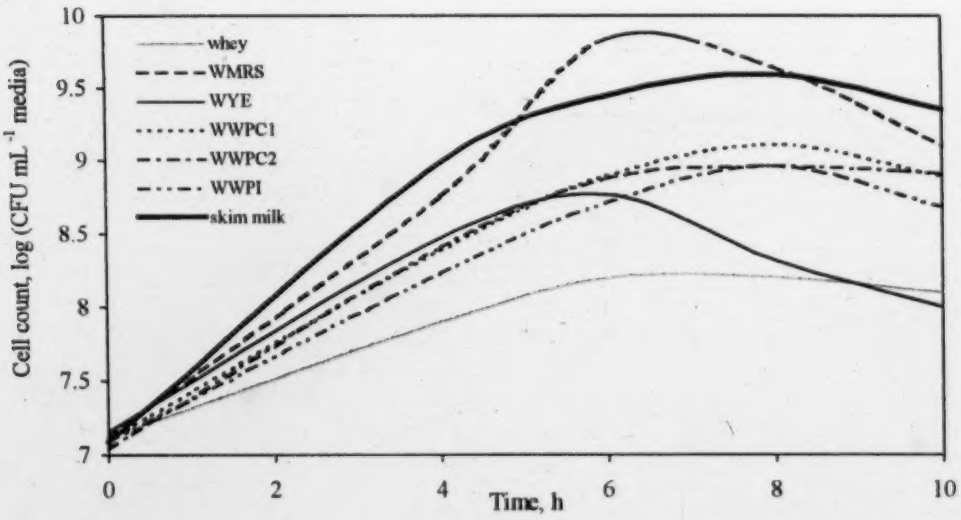
3.3. Results and discussion

3.3.1. The growth and lactic acid production of the culture

As expected, the supplementations of W or WP resulted in an increase of cell counts for all media. Figures 3-1a and 3-1b show the kinetics of culture growth in skim milk and in W or WP basal media supplemented with different adjuncts in comparison to the W and WP controls. While the highest cell count was obtained for the MRS supplemented whey medium (9.83 log CFU mL^{-1}), the culture experienced a sudden growth cessation after reaching its maximum. On the other hand, the skim milk cultivated culture reached the cell count of 9.59 log (CFU mL^{-1}), undergoing a slight decline afterwards.

Among the three whey protein supplements, WPC1 had the greatest positive effect on the growth, possibly due to its highest TCA-soluble nitrogen content (Table 3-2). The higher lactose content in the WPC1 likely had no effect (Bury et al., 2000), while the differences in the mineral content, especially phosphorus and calcium, were relatively minor. The supplementation of 0.2% yeast extract to W provided enough nutrients to support the growth of the culture to the same extent as in the whey protein supplemented media during the first four hours of cultivation. Generally, the addition of whey protein supplements extended the exponential growth phase, which lasted only six hours for basal media and basal media supplemented with YE and MRS.

a)



b)

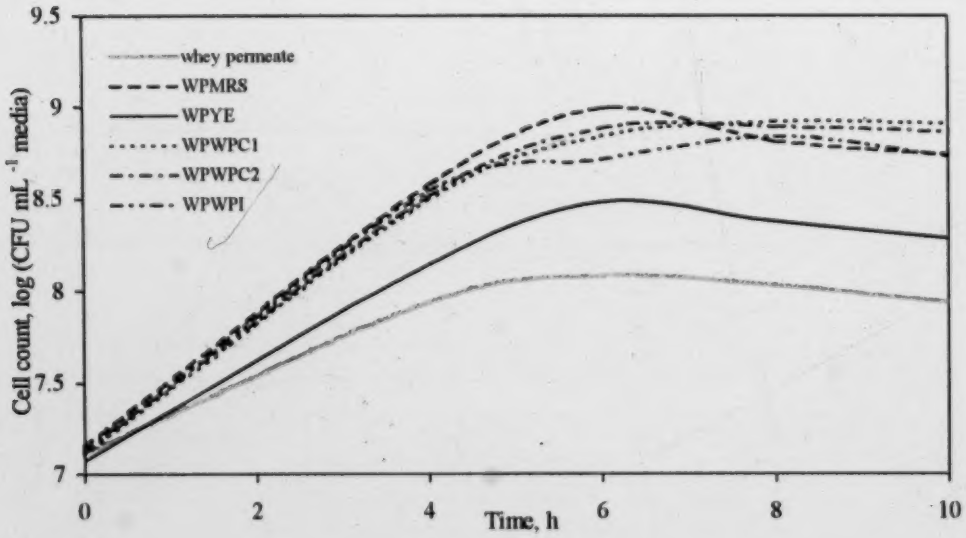


Figure 3-1: The growth kinetics of *Lb. bulgaricus* 11842 grown in (a) skim milk or whey and (b) whey permeate, with or without supplementation with different adjuncts.

For abbreviations see Table 3-1.

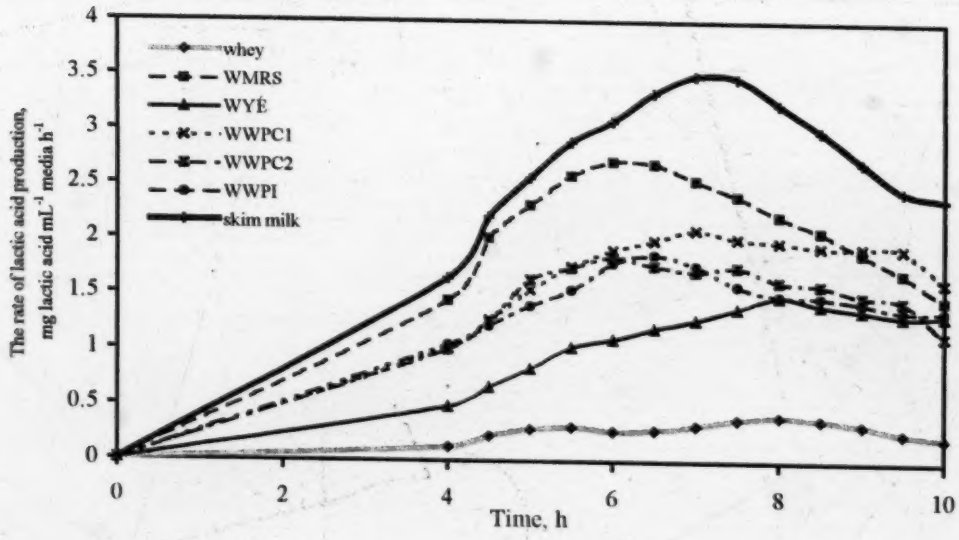
Figures 3-2a and 3-2b depict the rate of the lactic acid production of the culture cultivated in W and WP media. Comparing the lactic acid production rate to the growth kinetics (Figures 3-1a and 3-1b), it is noticeable that they had very similar patterns. The major difference appeared when the organism was cultivated in skim milk. The rate of lactic acid production reached its maximum after 8 hours at 3.5 mL of lactic acid mL^{-1} media h^{-1}, which differed significantly ($p<0.05$) from that produced in the culture grown in WMRS. All rates reached the maximum when the culture was in the stationary phase as expected.

Table 3-2: The compositional data of whey protein supplements (WPP) for whey and whey permeate media used in β-galactosidase production trials

COMPONENT	WPP*		
	WPC1	WPC2	WPI
Moisture, %	3.51±0.19 [a]	7.45±0.16 [b]	5.54±0.12 [c]
Ash, % on DM	4.04±0.17 [a]	2.92±0.03 [b]	2.82±0.04 [c]
Minerals, mg100 g^{-1} DM			
Calcium	558.81±17.75 [a]	553.38±9.48 [a]	476.89±5.84 [b]
Magnesium	59.03±2.40 [b]	58.90±2.12 [b]	77.26±1.21 [a]
Potassium	576.77±4.67 [a]	500.05±5.68 [c]	537.95±16.75 [b]
Sodium	348.89±9.18 [a]	180.21±4.76 [b]	131.27±2.56 [c]
Phosphate	348.21±24.65 [a]	349.63±13.58 [a]	211.55±7.37 [b]
Total protein, % of DM (N x 6.38)	73.66±0.43 [c]	83.15±0.23 [b]	92.36±0.53 [a]
TCA soluble nitrogen, % of DM (N x 6.38)	6.68±0.09 [a]	5.66±0.09 [b]	4.49±0.01 [c]
Lactose, % of DM	17.54±0.89 [a]	2.84±0.11 [b]	0.84±0.04 [c]

(Values with different letters in the same row are significantly different, $p<0.05$; * for abbreviations see Materials and Methods. The samples, in random order, were supplied by Promil, Novy Bydžov, Czech Republic; DMV USA, La Crosse, WI and Land-O-Lakes Inc., St. Paul, MN)

a)



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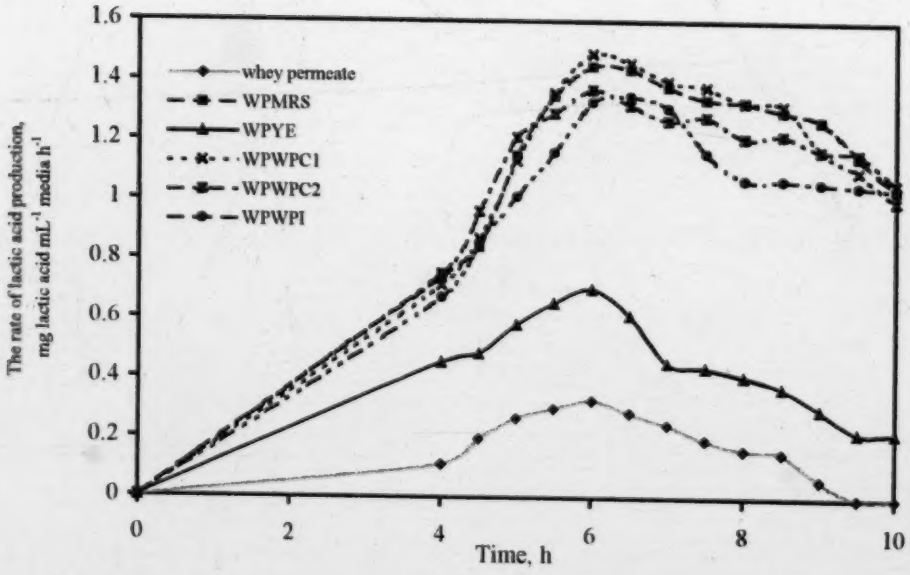


Figure 3-2: The rate of lactic acid production of *Lb bulgaricus* 11842 cultivated in (a) skim milk or whey and (b) whey permeate, with or without supplementation with different adjuncts.
For abbreviations see Table 3-1.

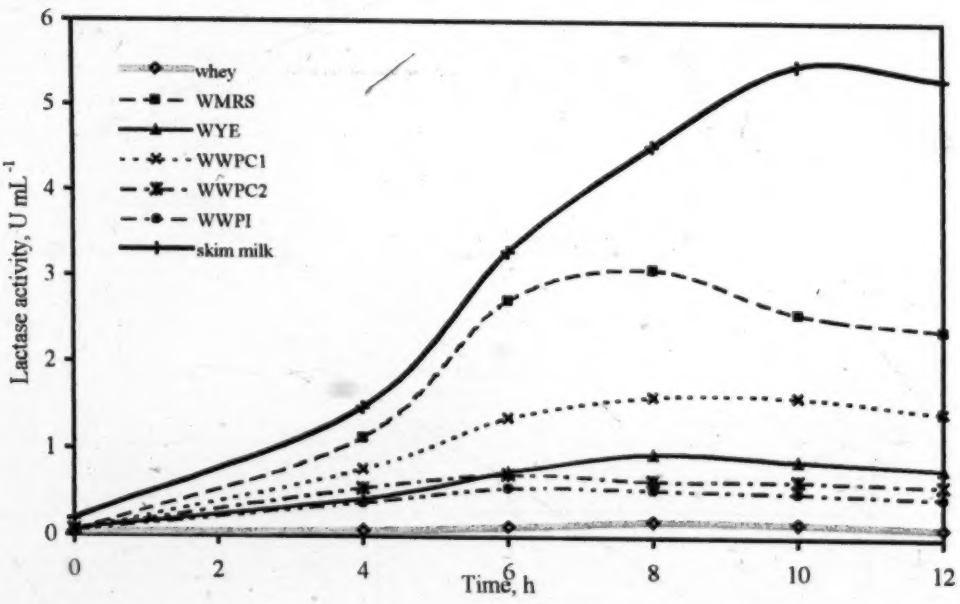
The cultures cultivated in WP media generally showed significantly lower ($p<0.05$) maximum rates of lactic acid production in comparison to the culture grown in whey based media. Even the addition of MRS to WP did not have the expected effect as when added to W. With the exception of WPYE resulting in significantly ($p<0.05$) lower rate, all other supplements to the WP resulted in very similar ($p>0.05$) rates of lactic acid production (Figure 3-2b).

Using formulas 3.2, 3.3 and 3.4 and the method of Roy et al. (1987), the kinetic parameters of the growth and lactic acid production were estimated and are presented in Table 3-3. For all media, the supplementation increased the specific growth rate. However, the statistical analysis revealed that there was significant difference ($p>0.05$) in the specific growth rate for milk and MRS supplemented basal media in comparison to all other media. Moreover, it appears that the specific growth rate of the culture cultivated in the media supplemented with WPP could be correlated to the TCA soluble nitrogen content constituted by amino acids and peptides (Table 3-2), confirming previous studies (Champagne et al., 1996; Leh and Charles, 1989). Thus, the WPC 1, despite its lowest total protein content, showed the greatest positive effect as in earlier investigations (Bury et al., 2000).

3.3.2. β-galactosidase activity as affected by the cultivation media

The β-galactosidase activity of the sonicated cultures clearly depended on enzyme production by the cells as affected by the kind of carbohydrate and amount of available nitrogen source present in the media. The highest lactase activity was obtained with the culture grown in skim milk (5.491±0.116 U activity mL^{-1} media). On the other hand,

a)



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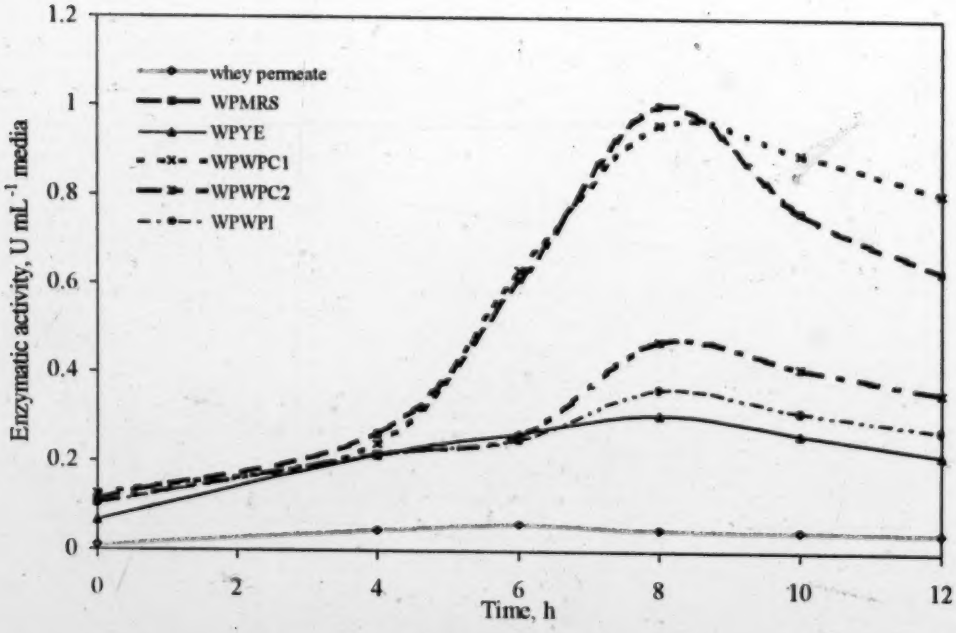

Figure 3-3: The β-galactosidase activity of *Lb. bulgaricus* 11842 cultivated in (a) skim milk or whey and (b) whey permeate, with or without supplementation with different adjuncts.
For abbreviations see Table 3-1.

MRS supplementation of W basal medium resulted in the highest specific growth rate and cell counts, yet the lactase activity (3.091±0.089 U activity mL^{-1} media) was 46% lower than when the culture was cultivated in skim milk. The synthesis of the β-galactosidase is governed by the transcription rate of *lac* genes, constituting the *lac* operon, and is enhanced or suppressed in the presence of an inducer or repressor (Wallenfels and Weil, 1970). The presence of glucose in MRS might cause the partial repression of the *lac* operon, resulting in the lower lactase activity of the culture. Furthermore, the lactase activities obtained in W or WP basal media supplemented with WPP (Figures 3-3a and 3-3b) differed significantly ($p<0.05$) and this could be correlated to the TCA soluble nitrogen content of the supplements (Table 3-2). However, none of the WPP supplementations resulted in enzyme activity comparable to that produced in skim milk.

To achieve higher biomass production - and indirectly increase the recovery of β-galactosidase - the energy derived through substrate level phosphorylation should be directed towards the synthesis of new cell material and, to a less extent, turnover reactions within the cell (Sinclair and Kristiansen, 1987). This tendency is clearly noticeable, if the lactase activity of cultures cultivated in different media (Figures 3-3a and 3-3b) are compared to P_α/P_β ratios (Table 3-3). The highest enzyme activity was achieved in the culture cultivated in skim milk, having the highest P_α/P_β ratio. On the other hand, cultures grown in whey and whey permeate basal media had low P_α/P_β ratios, resulting in low lactase activities. The correlational analysis revealed that the rate of lactic acid production was highly positively correlated with the buffering capacity and enzymatic activity, with R^2 coefficients of 0.906 and 0.866, respectively, indicating that

enzyme activity could be assessed indirectly through the volume of neutralizer employed. On the other hand, the correlation of growth rate to the rate of lactic acid production and enzymatic activity was low (R^2 = 0.326 and 0.332, respectively).

Table 3-3: Kinetic parameters of the growth and lactic acid production for *Lb. bulgaricus* 11842 and buffering capacity of the cultivation media

MEDIUM*	Specific growth rate, μ, h^{-1}	α mg CFU^{-1}	β mg CFU^{-1} h^{-1}	P_α/P_β	BC**, μmol lactic acid mL^{-1} media
Skim milk	0.747^{a}±0.056	$5.79x10^{-9}$	$9.00x10^{-10}$	3.28	18.67±0.12 [a]
Whey	0.416^{b}±0.025	$1.11x10^{-8}$	$2.56x10^{-9}$	0.92	10.24±0.02 [g]
WMRS	1.03^{a}±0.077	$1.39x10^{-9}$	$3.96x10^{-10}$	2.59	13.55±0.05 [b]
WYE	0.572^{b}±0.058	$6.59x10^{-9}$	$2.59x10^{-9}$	1.11	10.61±0.06 [f]
WWPC1	0.597^{b}±0.077	$9.18x10^{-9}$	$1.62x10^{-9}$	2.30	12.71±0.04 [d]
WWPC2	0.579^{b}±0.017	$1.26x10^{-8}$	$2.00x10^{-9}$	2.48	12.66±0.06 [d]
WWPI	0.553^{b}±0.054	$1.29x10^{-8}$	$2.02x10^{-9}$	2.42	12.47±0.06 [e]
Whey permeate	0.389^{b}±0.074	$1.11x10^{-8}$	$2.66x10^{-9}$	1.04	7.47±0.10 [i]
WPMRS	0.723^{a}±0.054	$8.69x10^{-9}$	$1.48x10^{-9}$	2.88	13.16±0.19 [c]
WPYE	0.541^{b}±0.066	$1.30x10^{-8}$	$2.33x10^{-9}$	2.19	8.95±0.11 [h]
WPWPC1	0.542^{b}±0.017	$1.07x10^{-8}$	$1.79x10^{-9}$	2.21	10.35±0.13 [g]
WPWPC2	0.533^{b}±0.027	$1.07x10^{-8}$	$1.76x10^{-9}$	2.22	10.11±0.11 [g]
WPWPI	0.506^{b}±0.028	$1.25x10^{-8}$	$2.00x10^{-9}$	2.19	9.98±0.17 [g]

[a-h] Values with different letters in a column are significantly different as determined by a t-test, $p<0.05$; mean±SD; * for abbreviations see Table 1. ** BC – buffering capacity; formulas 3.1. and 3.2. used to calculate the data.

3.3.3. Spent skim milk reprocessing

The β-galactosidase activity of a given microorganism depends on the characteristics of a medium. To maximize the enzyme activity, a rich medium, as well as the absence of a *lac* operon repressor such as glucose, are necessary. For the feasibility of β-galactosidase production using thermophilic LAB, it appears preferable to use skim milk as the medium of choice rather than enrichment of whey or whey permeate basal media with suitable adjuncts. Furthermore, the lactase activity is maximized if the pH of the well-buffered medium is controlled by a suitable neutralizer.

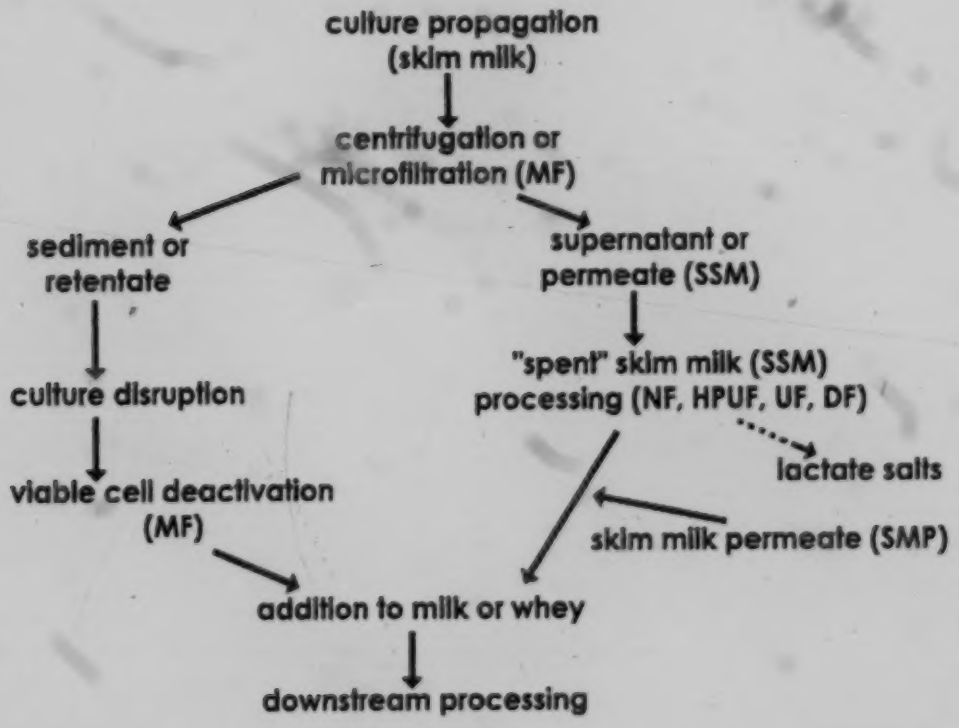

Figure 3-4: The flow diagram of a proposed process for the lactose hydrolysis in dairy products based on in-house production of β-galactosidase from *Lb. bulgaricus* 11842 cultivated in skim milk including reuse of the fermentation medium.

In this regard, a semi- pilot plant process for the β-galactosidase production was tested with the complementary focus on the utilization of spent skim milk (SSM), used as the fermentation medium for the enzyme production. As seen from Table 3-4, the major difference between SSM and commercial skim milk (SM) was the content of sodium and ash, being 3.5 and 7.1-fold (respectively) higher in SSM due to the use of sodium hydroxide as neutralizer during fermentation. By HPUF of the SSM and addition of skim milk permeate (SMP) to the spent skim milk retentate (SSMR), a 27.4% decrease in the sodium content of the recombined product (RP) was obtained. This implies that by employing different UF conditions (temperature, pressure, membrane type, VCR) and adding regular SMP to adjust the total solids content of RP, it could be possible to obtain a product suitable for further use. Fermentation of the RP by the sour cream or yogurt cultures showed no unusual problems. Sensory properties of the two products prepared from the RP were cursorily examined by trained experts. The major defects were brownish color, likely originating from Maillard reaction during the media sterilization, and saltiness. However, the product simulating sour cream (SCRP) was considered acceptable by the evaluators, indicating further possibilities for product and process optimization.

A process for the β-galactosidase production with the reuse of SSM can be visualized as shown in Figure 3-4, with either bactofugation or microfiltration envisioned as suitable technological steps for cell separation. Similarly, the removal of the excess sodium and lactate can be presumably improved by a more efficient membrane process application.

Table 3-4: Compositional analysis of spent skim milk (SSM), spent skim retentate (SSMR), skim milk (SM), skim milk permeate (SMP) and 1:1 SSMR and SMP recombined product (RP)

COMPONENT	SSM	SSMR	SM	SMP	RP
Total solids, %	7.44	12.74	8.45	2.01	7.42
Ash, %	1.33	1.64	0.38	0.26	0.98
Potassium, mg L^{-1}	1448.8	1926.1	1525.5	1055.5	1410.5
Calcium, mg L^{-1}	887.5	1422.5	1017.0	47	802.0
Sodium, mg L^{-1}	2727.3	3617.6	385.9	308.5	1980.3

There has been a great deal of skim milk permeate (SMP) on the market lately (Lankveld, 1995) and the SMP addition could present a new desirable way for the total solids and sodium content adjustment in the recombined product, as well as an opportunity for additional SMP utilization. In a parallel investigation, preliminary sensory analysis of several lactose hydrolyzed products, made with the β-galactosidase preparation obtained according to the proposed scheme, revealed that the change of product sweetness was the main sensory effect observed after 24 hour lactose hydrolysis treatment at 7°C (Vasiljevic and Jelen, 2000b).

3.4. Conclusions

A recent feasibility study of in-house production of the crude β-galactosidase preparation from thermophilic LAB showed that the process could be technically and economically feasible even if significant volumes of growth media were to be used for the production of the enzyme (Bury, and Jelen, 2000). However, this study disregarded

the problem of utilization of the spent fermented whey based medium after culture harvesting. The present study illustrated that the use of skim milk for lactase production could enable an efficient in-house production of the enzyme for lactose hydrolysis, thus decreasing the volume of the media to be reprocessed. The fastidious nature of LAB, requiring rich medium for the growth and lactase activity, as well as the presence of *lac* operon repressor, appeared to result in lower lactase activity obtained from the whey and whey permeate even with substantial supplementation. The extensive enrichment of whey or whey permeate basal media may be economically unfeasible due to the cost of the supplements and because the further utilization of such spent media appears doubtful. Skim milk as a growth medium would result in the highest lactase activity and it would provide an opportunity for further reprocessing into valuable consumer products.

3.5. References

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Chapter 4*

Lactose Hydrolysis in Milk as Affected by Neutralizers Used for Preparation of Crude β-Galactosidase Extracts from *Lactobacillus bulgaricus* 11842

4.1. Introduction

Lactose hydrolysis in milk and other dairy products by the enzyme β-galactosidase (EC 3.2.1.23) is of considerable interest to the dairy industry. It has been estimated that over 70% of world's inhabitants suffer from either lactose maldigestion or intolerance (de Vrese et al., 2001). A suitable, economically feasible process for lactose hydrolysis may alleviate this problem but also diminish some technological difficulties associated with lactose crystallization in products such as ice cream (Zadow, 1993) and whey spreads (Patocka and Jelen, 1988). It may also open new possibilities for the utilization of whey through production of sweeteners and development of novel products from lactose hydrolyzed whey (Zadow, 1993).

The traditional sources of β-galactosidase are of microbial origin, mainly yeasts and molds (Mahoney, 1997). More recently, lactic acid bacteria (LAB) have become a scientific focus of studies for two particular reasons: a) lactose maldigesters may consume some fermented dairy products with little or no adverse effects; and, b) LAB are generally regarded as safe so the enzymes derived from them might be used without extensive purification. Among all LAB, the strains used in yogurt production, consisting

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of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, have been the most extensively studied. Their β-galactosidases were characterized and different processes, such as batch and immobilization techniques, were explored for the possible industrial use. Jelen (1993) suggested that specific microbial strains which are high β-galactosidase producers could be used for lactose hydrolysis after cell disruption but with minimum additional purification. More recently, Vasiljevic and Jelen (2001) proposed a process for lactose hydrolysis in dairy systems using a crude cellular extract (CCE) from the *Lactobacillus bulgaricus* 11842 culture, with subsequent utilization of the fermentation medium used for culture propagation.

The use of CCEs may be economically feasible for lactose hydrolysis due to elimination of the high costs of the enzyme purification; however, its application in the dairy industry may require much additional research. The activity and stability of β-galactosidase is strongly influenced by the enzyme origin and environmental conditions of enzyme production and/or the hydrolysis process, such as temperature and pH optima as well as a presence of activators or inhibitors.

Divalent cations such as magnesium and manganese may enhance the β-galactosidase activity, while monovalent cations may have positive or negative effect (Pivarnik and Rand, 1992; Garman et al., 1996; Kreft and Jelen, 2000). Other media components, such as casein, whey proteins and lactose, also may have a significant effect on the activity and/or stability of β-galactosidase from various sources, resulting in variable outcomes (Mahoney and Adamchuk, 1980; Greenberg et al., 1985). Therefore, the lactose hydrolysis in a complex system such as milk may differ greatly from that conducted in a simple buffered lactose solution. The impact of neutralizing agents used

for the pH maintenance during fermentation on the activity and stability of the enzyme, as well as sensory properties of the lactose hydrolyzed dairy products using the CCE approach need to be fully characterized. The use of CCEs instead of purified β-galactosidase preparation may complicate the final outcome of the hydrolysis due to the presence of other microbial enzymes, which may interfere with lactose hydrolysis.

The aims of this study were to evaluate the effects of three different neutralizers, used for pH maintenance during the cultivation of *Lactobacillus bulgaricus* 11842, on: a) preparation of crude β-galactosidase extracts for lactose hydrolysis; b) cell viability and proteolytic activity of CCEs; and c) kinetics of lactose hydrolysis in skim milk.

4.2. Materials and Methods

4.2.1 Materials

Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842 (Lb. 11842), a thermophilic strain of a common yogurt microorganism, was obtained from the Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada. The ONPG (o-nitrophenyl-D-galactopyranosyde) reagent was purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). All other analytical grade chemicals were obtained from Fisher (Fisher Scientific, Pittsburgh).

4.2.2 Preparation of crude cellular extracts

The Lb. 11842 culture was propagated every second day in sterile skim milk. Approximately 1 mL of the culture was used to inoculate 150 mL of sterile skim milk and incubated at 43±1°C with circular rotation at 150±25 RPM in a controlled environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ) for 10 hours. From this culture, 50 mL were added to 1.5 L of fermentation medium, prepared by sterilization of commercial fluid skim milk (Dairy World Foods, Vancouver, BC) at 121°C for 15 min. The fermentations were carried out in a 2 L fermentor (New Brunswick Scientific Co., New Brunswick, NJ). The temperature was controlled by placing the fermentor in a water bath at 43±0.2°C. The pH was maintained at pH 5.6±0.1 by the automatic addition of a 2M hydroxide (ammonium, sodium or potassium) using a peristaltic pump (Watson-Marlow, Cornwall, UK) hooked to a pH controller (Omega Engineering Inc., Stanford, CO).

All fermentations were terminated after 10 hours. The cells were collected by centrifugation (Beckman model J2-21, Beckman Coulter Inc, Fullerton, CA) at 4000 *x g* at 4°C for 10 min and kept frozen at -35°C till needed. The obtained frozen cell paste (approx. 14% dry matter) was thawed at 37°C in a water bath and approximately 80 mL was transferred into a bead mill (Dyno-Mill type KDL, Impandex Inc., Maywood, NJ) for the disruption using 0.2 to 0.3 mm glass beads for 2 min at 4°C. The resulting material, referred to as crude cellular extract (CCE), was used in all lactose hydrolysis experiments without further purification.

4.2.3 The effect of neutralizers on cell disruption and enzyme activity

The efficiencies of the centrifugation and cellular disruption operations were determined by viable plate counts before and after disruption using standard serial dilution techniques with 0.1% peptone (Bacto-peptone, Difco Laboratories, Becton-Dickenson Detroit, MI) in phosphate buffer, pH 7. The plates were poured with MRS containing 1.5% agar prepared according to the manufacturer's instructions. Plates were incubated aerobically at 43°C and counted after 48 hours. The efficiency of disruption was estimated from the differences in bacterial numbers expressed as colony forming units (CFU mL^{-1}) before and after disruption.

The β-galactosidase activity of the CCE was assessed using o-nitrophenyl-ß-D-galactopyranoside (ONPG) as the substrate. The samples taken for this analysis (500 µL) were diluted in phosphate buffer, pH 7, and the β-galactosidase activity was determined as described previously (Shah and Jelen, 1991; Vasiljevic and Jelen, 2001). The concentration of o-nitrophenol (ONP) released was determined from the absorbance at 420 nm, using the standard calibration curve (Spectronic 21, Bausch & Lamb, USA). The enzymatic activity was expressed as units of β-galactosidase activity per mL of concentrated cells. A unit of activity was defined as that liberating 1 µmol of ONP from ONPG per minute per mL of wet cell paste under experimental conditions described above. All solutions were cooled in ice water. Experiments were replicated and all analyses were carried out at least in duplicate.

4.2.4 Lactose hydrolysis in the reaction mixtures

Pasteurized fluid skim milk (Lucerne Milk Department, Edmonton, AB, Canada) was used to produce the reaction mixtures for lactose hydrolysis. The CCEs were washed out from the bead mill with appropriate amounts of the skim milk, resulting in the final concentration of 1 or 2% (v/v). The reaction mixtures were centrifuged again under conditions described above, for removal of viable cells, glass beads and cell debris, divided into 5 mL samples and held at 7, 20, 37, 55 or 65°C for 6 to 10 hrs depending on the temperature. The progress of lactose hydrolysis was monitored by change of freezing point with an Advanced Cryomatic Milk Cryoscope (model 4C2, Advanced Instruments Inc., Norwood, Mass.).

Theoretically, the change of freezing point of 281 m°H would represent 100% lactose hydrolysis (Kreft et al., 2001). Additionally, the changes of lactose, glucose and galactose concentration during hydrolysis were also monitored by high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) equipped with a refractometer. Separation took place in an Aminex HPX-87H, 300 x 7.8 mm ion exclusion column (Biorad, Hercules, CA) equipped with a guard and ion-exchange column (Phenomenex, Torrence, CA) at 35°C with 5 mM H_2SO_4 as mobile phase at flow rate of 0.6 mL min^{-1}.

The quantification of sugars was inferred from the standard curves, obtained using external standard solutions of predetermined concentrations. Samples for HPLC determinations were diluted in 100% ethanol (1:9 ratio) and centrifuged for protein removal. The supernatant was filtered through 0.22 µm Durapore membrane filter (Millipore, Bedford, Mass.), vacuum dried at 20°C till dryness, and the dry residues were dissolved in distilled water to the original volume. The degree of lactose hydrolysis was

calculated as a mass fraction and expressed as the degree of conversion, X (g g^{-1}), using the following equation (Mahoney, 1997):

$$X = \frac{[glu]+[gal]}{[lactose]} \qquad \text{Formula 4.1.}$$

where: [glu] and [gal] are the concentrations of glucose and galactose formed during the lactose hydrolysis (g L^{-1}), respectively; and [lactose] is the initial lactose concentration in skim milk (g L^{-1}). The pH change in milk during lactose hydrolysis treatment was monitored every hour by a pH meter (model 701A, Orion research Inc, Cambridge, Mass), and viable cell counts were determined at the beginning and end of the lactose hydrolysis runs using the method described above.

To predict the performance of the CCE in possible industrial conditions, an attempt was made to estimate kinetic parameters k_{cat} and K_m for the crude β-galactosidase extract. In this regard, an integrated graphical method was deployed to estimate the kinetic constants directly from a linear plot using data from a replicated single experiment instead of graphical differentiation, usually used in kinetic determinations. The parameters were estimated from the following equation (Halwachs, 1978):

$$\frac{t}{X} = \frac{K_m}{V_{max}} \cdot \left(\frac{1}{X} \ln \frac{1}{1-X} - 1\right) - \frac{S_0 + K_m}{V_{max}} \qquad \text{Formula 4.2.}$$

where: X – degree of conversion, g g^{-1}; $V_{max} = k_{cat} \cdot E_0$ – maximum rate of reaction, μM min^{-1}; k_{cat} – catalytic constant, μmol U^{-1} min^{-1}; E_0 – initial enzyme concentration, U L^{-1}; K_m – Michaelis-Menten constant, mM; and S_0 – initial lactose concentration, mM.

From Formula 4.2., it is possible to predict the time, t (min), to achieve a desirable degree of lactose conversion if other parameters are known or any other parameter if t and X are known.

4.2.5 Determination of proteolysis

The proteolytic activity of the CCE in the milk over the course of the lactose hydrolyzing runs was assessed by peptide mapping of the hydrolyzed products, performed using a Sephasil reverse phase peptide column (Pharmacia Biotech), 4.6 mm x 100 mm packed with 5 mm diameter carbon 18 bound sterophil beads. Peaks were a function of absorbance observed by an ultra violet/visible wavelength detector (Shimadzu SPD-10A) operating a wavelength of 220 nm. Total peak area was obtained by integration of all the peaks observed. Elution was accomplished using a gradient of two mobile phase solvents: 0.1 % trifluoroacetic acid in deionized water and acetonitrile (HPLC grade). The concentration of acetonitrile was increased linearly over twenty minutes from 0 to 60%. Total flow rate was constant at 1 mL min^{-1}. Columns were rinsed with 60% acetonitrile for ten minutes and the baseline was stabilized for an additional 10 minutes between sample elutions. Samples (skim milk diluted in deionized water 1:4, passed through a 0.22 µm Millipore filter) were analyzed in duplicate. Temperature was maintained constant at 22°C. The change of peptide profile was expressed as relative proteolytic activity, Rpa, %, calculated from the following equation:

$$Rpa,\% = \frac{TPE_e - TPE_b}{TPE_b} \cdot 100 \qquad \text{Formula 4.3.}$$

where: TPE_e and TPE_b are total peak area at the end and beginning of hydrolysis, respectively.

4.2.6. Statistical analysis

All experiments were replicated and all analyses were carried out at least in duplicate. Unless indicated otherwise, results are expressed as average ± standard deviation (SD) using all available data. Statistical significance of differences ($p < 0.05$) was determined by ANOVA and t-test where appropriate using the Excel Analysis ToolPak (Microsoft Office 2000).

4.3. Results and Discussion

4.3.1 The effect of neutralizers on β-galactosidase activity and cell counts

This study extends the evaluation of individual steps in the proposed process (Vasiljevic and Jelen, 2001) for the production of lactose hydrolyzed dairy materials using the CCE from a disrupted culture of *Lb. bulgaricus* 11842. One such step that could have a significant effect on the process and the final product is the cultivation of the enzyme producing culture, including the selection of the neutralizing agent.

The CCE obtained using NH_4OH as the neutralizer during the culture growth had significantly ($p<0.05$) higher β-galactosidase activity than the other two CCEs (Table 4-1). This could be due to a specific response of the culture to the addition of NH_4OH during cultivation rather than increased bacterial growth.

Noticeably, but not dramatically, the cell counts obtained during NH_4OH fermentation were 0.18 and 0.34 log CFU mL^{-1} higher than those of the NaOH and KOH fermentation, respectively. The cell collection by centrifugation resulted in further cell count increases between 0.66 and 0.75 log CFU mL^{-1} for all three cultivations (Table 4-1). The bead milling proved to be a very efficient tool for cell disruption. It produced an average cell count decrease of 2.5 log CFU mL^{-1}, representing an efficiency of disruption of 99.5%. Prior to hydrolysis, viable cell counts ranged from 4.34 to 4.76 log CFU mL^{-1} as a result of dilution of the CCE with skim milk and centrifugation afterwards.

Table 4-1: The enzyme activity and cell counts (means ± standard deviation, n = 4) during production of the CCE from the cell paste obtained by fermentation of *Lactobacillus bulgaricus* 11842 in skim milk using three different neutralizers.

Neutralizer	β-gal activity, U mL^{-1}	Cell count, log CFU mL^{-1}			
	Disruption	Cultivation	Centrifugation	Disruption	Start of hydrolysis
NaOH	74.28 ± 10.70	9.19 ± 0.01	9.91 ± 0.02	7.34 ± 0.02	4.56 ± 0.03
KOH	68.79 ± 5.96	9.03 ± 0.04	9.69 ± 0.03	7.15 ± 0.02	4.34 ± 0.03
NH_4OH	118.47 ± 14.97	9.37 ± 0.03	10.12 ± 0.01	7.65 ± 0.03	4.76 ± 0.02

The remaining viable cells contained in the CCE may present an opportunity for undesirable growth, which could affect the quality of a final product. Milk, as a suitable medium, provides all necessary nutrients for dairy cultures and the availability of glucose

from the lactose hydrolysis and possible products of proteolytic activity would further create suitable conditions to support active growth of undesirable microorganisms.

As expected, cell counts at the end of the lactose hydrolysis experiments increased for all CCEs at 20 and 37°C in comparison to the initial counts. The increase in cell count was the highest (2.78 log CFU) after addition of 2% of NH_4OH CCE resulting in the pH decrease of 0.56 and final pH 6.18 at the end of the 6-hour lactose hydrolysis runs at 37°C. Generally, the addition of smaller amounts of the CCE produced smaller change of log CFU and pH (Appendix, Figure A-1 and A-2). No growth occurred at 7°C, with a slight decrease in cell counts (0.2 to 0.3 log CFU mL^{-1}), while the cell counts were substantially reduced at the end of lactose hydrolysis (< 10^2 CFU mL^{-1}) at 55 and 65°C. The change of pH during hydrolysis at 7 and 55°C was slight and ranged from 0.1 to 0.3 pH units for all CCEs.

4.3.2. Effectiveness of lactose hydrolysis in skim milk

The rate of lactose hydrolysis may be determined by several methods; however, the determination of freezing point depression (FPD) was chosen for its simplicity, accuracy and reproducibility (Chen et al., 1981; Kreft and Jelen, 2000). HPLC was employed as a confirmatory method. The results obtained by HPLC, expressed as the degree of conversion, g g^{-1}, were plotted against the freezing point depression and the correlation was best described by the binomial curve as:

$$X = -2.162 \cdot 10^{-6} \cdot \Delta FPD^2 + 3.891 \cdot 10^{-3} \cdot \Delta FPD \qquad \text{Formula 4.4}$$

where: X represents the degree of conversion, g g^{-1} (see Materials and Methods); and ΔFPD is the change of freezing point depression, reported in millidegree Hortvet, m°H. The correlation coefficient was 0.9966. The results obtained for the empirical 100% lactose hydrolysis were slightly higher than the theoretical value of 281 m°H, most likely due to formation of low molecular weight compounds, produced during the growth of viable cells remaining after disruption and centrifugation, or from other enzymatically mediated reactions such as proteolysis.

Results expressed as the rate of conversion in time showed the typical hyperbolic relationship with the exception of the curve for the lactose hydrolysis runs performed at 65°C (Figures 4-1, 2 and 3). Noticeably, there was no change in the freezing point depression after 1 hr when NaOH and NH_4OH CCEs were used, which indicated β-gal inactivation. The cessation of β-gal activity of KOH CCE occurred at 1.5 hr, resulting in the highest degree of lactose hydrolysis at 65°C (Figure 4-2), even though the cell paste had the lowest enzyme activity (Table 4-1). This could be due to the stabilizing effect of potassium on this β-galactosidase (Flores et al, 1996; Kreft and Jelen, 2000). Flores et al. (1996) also reported that ammonium ion was more efficient than Na or K ion in protecting *Kluyveromyces lactis* β-gal from thermal inactivation; however, it appeared in our study that the *Lb. bulgaricus* β-gal was deactivated as fast at 65°C in the presence of ammonium ion as when Na ion was present. At all other temperatures, the degree of hydrolysis depended mainly on the activity of the cell paste after disruption and the concentration of the CCE added, being the highest for NH_4OH paste at all temperatures (Figures 4-1, 2 and 3). Surprisingly, the addition of 2% CCEs from NaOH cultures

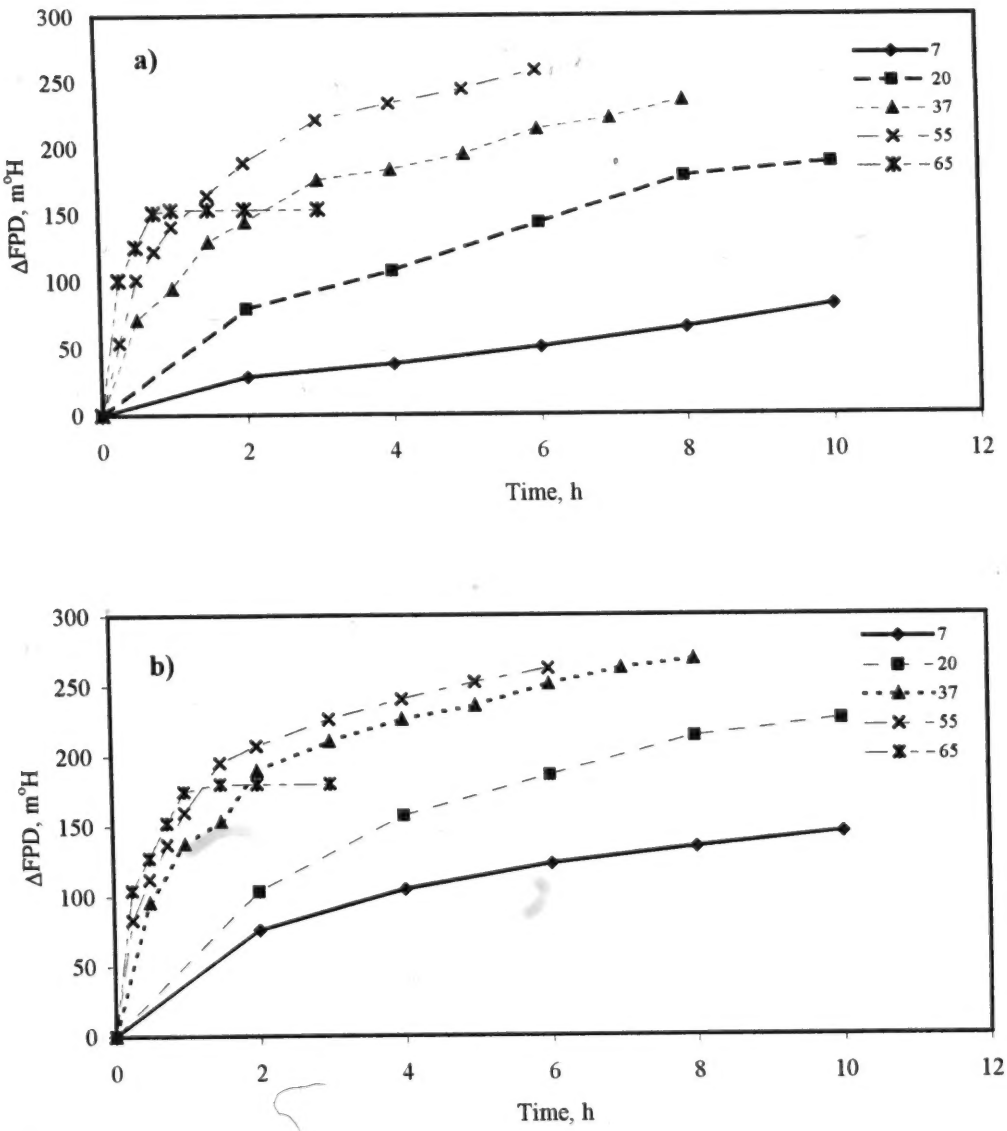

Figure 4-1: Development of freezing point depression change (ΔFPD) during lactose hydrolysis in skim milk at 7, 20, 37, 55 or 65°C using a) 1% and b) 2% (v/v) of a CCE originating from fermentation of *Lactobacillus bulgaricus* 11842 in skim milk using NaOH as neutralizer

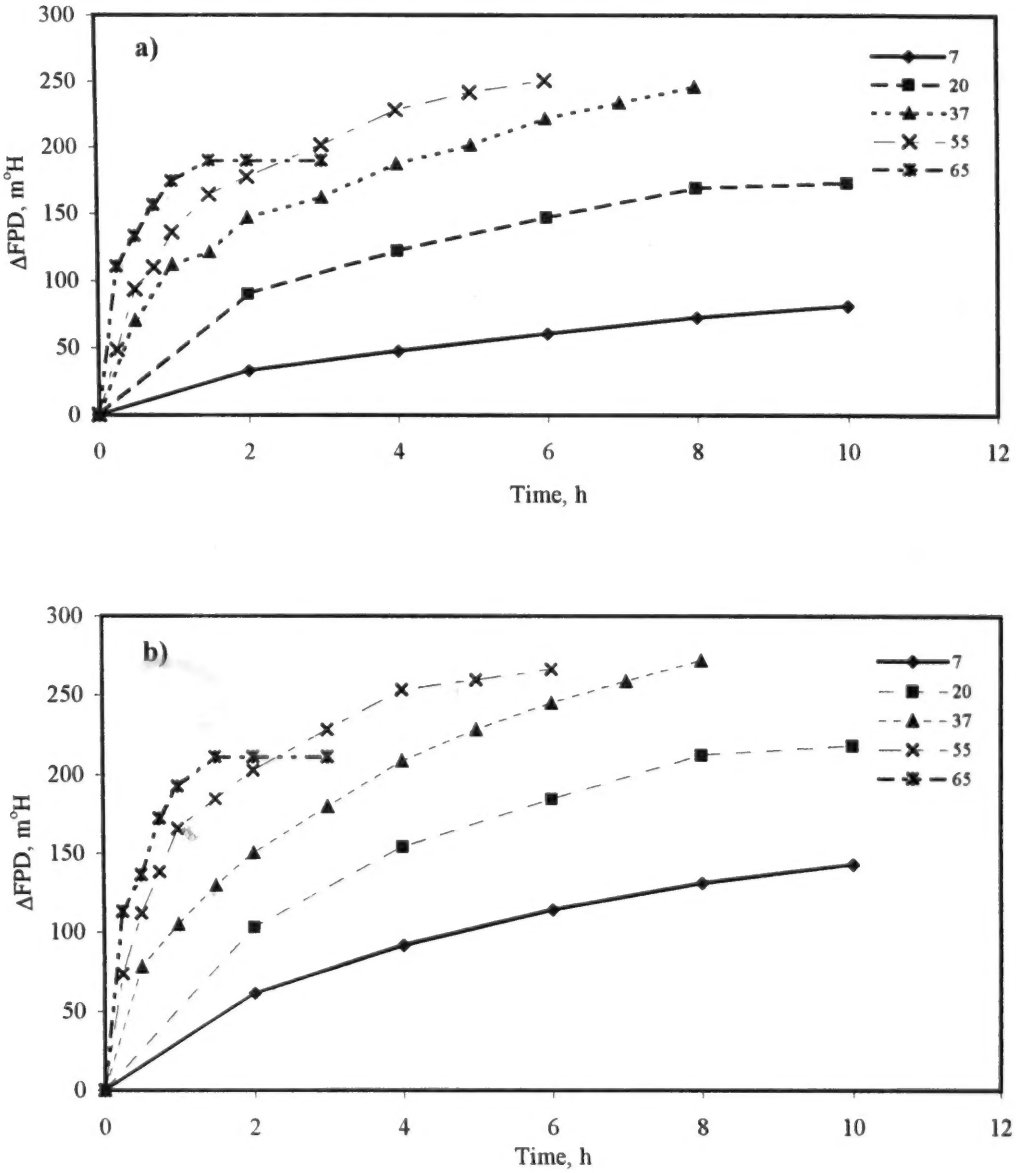

Figure 4-2: Development of freezing point depression change (ΔFPD) during lactose hydrolysis in skim milk at 7, 20, 37, 55 or 65°C using a) 1% and b) 2% (v/v) of a CCE originating from fermentation of *Lactobacillus bulgaricus* 11842 in skim milk using KOH as neutralizer

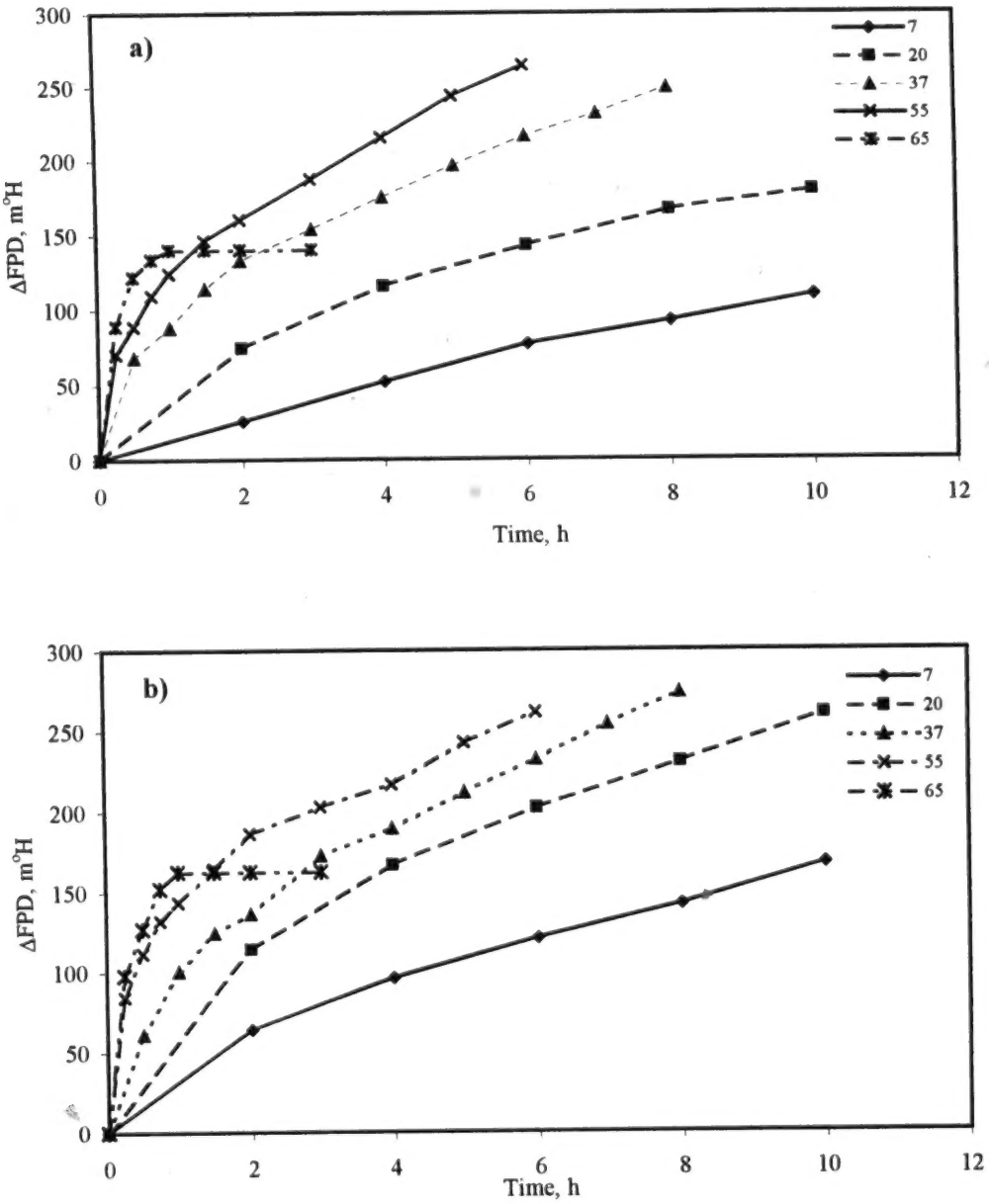

Figure 4-3: Development of freezing point depression change (ΔFPD) during lactose hydrolysis in skim milk at 7, 20, 37, 55 or 65°C using a) 1% and b) 2% (v/v) of a CCE originating from fermentation of *Lactobacillus bulgaricus* 11842 in skim milk using NH_4OH as neutralizer

resulted in an unexpectedly low rate of lactose hydrolysis at 55°C. As presented in Figure 4-1b, the rate curves at 37 and 55°C were similar, which might have been caused by enhanced β-gal deactivation, since Na has been shown to have a negative effect on this enzyme (Kreft and Jelen, 2000). The rate of lactose hydrolysis increased at higher temperatures for all the CCEs indicating that the rate constants followed the Arrhenius kinetics.

4.3.3 Determination of kinetic parameters

The establishment of reliable kinetic parameters for lactose hydrolysis by the crude β-galactosidase extract in skim milk poses several difficulties mainly due to complexity of the system. Besides being a well-buffered solution, which enhances the activity and stability of the enzyme, skim milk contains different components, which may have different effects on enzyme characteristics. The stability of β-galactosidase is typically 50 to 100 times greater in skim milk than in buffered lactose solutions mainly due to casein and lactose acting in concert (Mahoney and Adamchuk, 1980; Mahoney and Wilder, 1989; Chang and Mahoney, 1989). The presence of divalent and monovalent ions may have positive or negative effect, depending on the enzyme origin (Voget et al., 1994; Flores et al., 1996; Garman et al., 1996).

The graphical differentiation method often used for the determination of kinetic parameters requires plotting the initial velocity rates against different substrate concentrations. The change of milk composition by addition or removal of lactose may produce results which would not describe the enzyme properties and behavior in the real system appropriately. Therefore, the integrated graphical method as described by

Halwachs (1978) was employed to estimate the parameters in Michaelis-Menten type kinetics from one replicated data set using the addition of 1% CCEs, which allowed comparisons with previous studies (Table 4-2). Similar evaluation of the 2% CCE runs (Appendix, Table A-1) showed broad agreement after accounting for the different enzyme concentrations used.

Table 4-2: The estimation of kinetic parameters in Michaelis-Menten type kinetics, k_{cat} and K_m, for the lactose hydrolysis in skim milk at different temperatures using 1% (v/v) CCE produced with three different neutralizers

Temperature °C	Na		K		NH_4	
	k_{cat}, μmol U^{-1} min^{-1}	K_m, mM	k_{cat}, μmol U^{-1} min^{-1}	K_m, mM	k_{cat}, μmol U^{-1} min^{-1}	K_m, mM
7	43.7 ±0.87	172.7 ±19.55	42.4 ±1.68	147.7 ±9.1	43.3 ±3.07	102.9 ±20.28
20	104.5 ±0.85	198.6 ±0.64	106.4 ±4.88	138.0 ±1.4	104.86 ±8.53	158.5 ±1.65
37	312.2 ±4.19	139.6 ±1.13	323.7 ±6.73	148.8 ±3.9	340.3 ±18.54	178.2 ±3.36
55	611.7 ±60.47	141.5 ±0.45	625.7 ±19.81	144.7 ±2.9	592.1 ±56.12	168.0 ±7.06

(means ± standard deviation, n = 4)

The above kinetic parameters could be used to predict enzyme efficiency in industrial conditions (Bernal and Jelen, 1989) and enable the prediction of the time needed for a specific degree of lactose hydrolysis. The K_m values ranged from 102.9 to 198.6 mmol L^{-1}, but most frequently between 140 and 150 mmol L^{-1}, close to the initial lactose concentration of 140 mmol L^{-1}. The values were slightly higher than those reported earlier (Bernal and Jelen, 1989); however, this may be influenced by different methods of determination, the enzyme origin as well as by the fact that the previous study was performed in a model system.

The Michaelis-Menten kinetics model used in this study disregarded the influence of galactose and glucose inhibition on the rate of lactose hydrolysis (Itoh et al., 1980). The catalytic constant, k_{cat}, was clearly temperature dependent following the Arrhenius kinetics (Figure 4-4), as reflected in the rates of lactose hydrolysis measured (Figure 4-1, 2, 3).

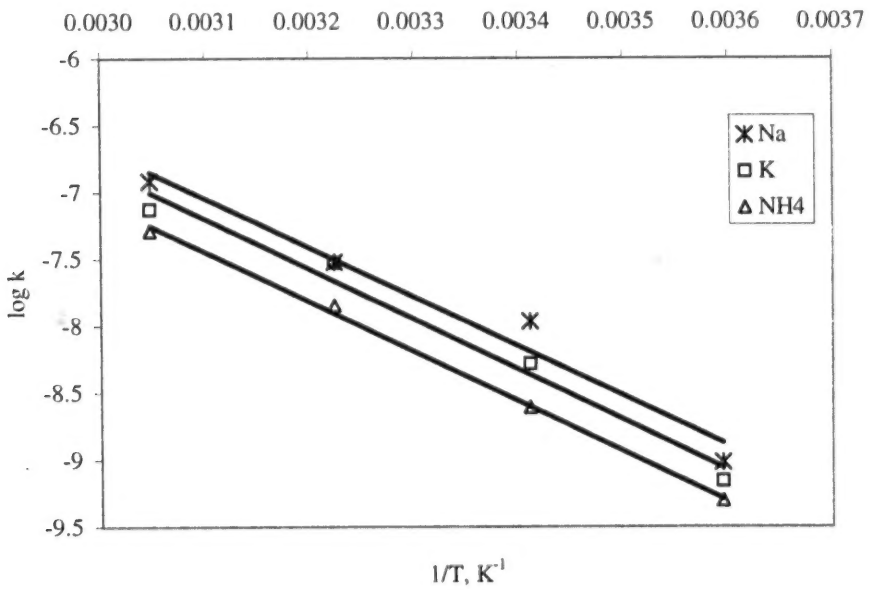

Figure 4-4: Arrhenius plot for the β-galactosidase containing CCEs obtained with NaOH, KOH or NH_4OH neutralizers during the lactose hydrolysis in skim milk by the 1% (v/v) addition of CCEs

However, there was no significant difference ($p>0.05$) among the catalytic constants (k_{cat}) for the different neutralizers (Table 4-2; Figure 4-4). The calculated values for the energy of activation were 42.8±5.3, 43.5±0.7 and 42.7±2.2 KJ mol^{-1} for Na, K and NH_4 CCE, respectively. These values are in agreement with the data for *Kluyveromyces fragilis* β-galactosidase (Santos et al., 1998). The catalytic efficiency and specificity of the enzyme, presented as the ratio V_{max}/K_m, showed the highest values at 55°C and ranged between

2.97 and 4.00 x 10^{-3} with no significant difference ($p>0.05$) among the three CCEs. These values were in broad agreement with the V_{max}/K_m value of 3.5 x 10^{-3} reported for β-galactosidase from *Escherichia coli* (Whitaker, 1994).

4.3.4 Determination of proteolysis during lactose hydrolysis

Lactobacillus bulgaricus grows well in milk mainly due to developed proteolytic system highly specific for caseins (Analia et al., 1993). An inefficient removal of cell debris and viable cells from the reaction mixture prior to the lactose hydrolysis in skim milk may result in high concentrations of proteolytic enzymes in the CCE. This in turn may induce bitterness or other off-flavours originating from the resulting peptides, sometimes even gelation of the milk (Modler et al., 1993), as well as affecting the stability of the β-galactosidase.

The proteolytic activities of the three CCEs were evaluated using peptide mapping by HPLC. As seen from Figure 4-5, the proteolytic activity was the lowest for the CCE from NH_4 fermentation for both concentrations used in the study. On the other hand, the addition of the 1 or 2% (v/v) NaOH CCE resulted in almost five fold increase in the proteolytic activity compared to the NH_4 or K CCE at the apparent temperature optimum of about 40°C. Generally, the proteolytic activity increased with the increased concentration of the CCEs and was the highest at moderate temperatures (20 and 37°C). The proteolytic activity of all three CCEs used at 55°C in the 1% (v/v) concentration were very low, most likely due to deactivation of the proteolytic enzymes at this temperature. Thus, it appears that conducting the lactose hydrolysis at 55°C with any of

the CCEs should minimize the undesirable changes caused by both the growth of the viable cells and the unwanted proteolytic activities.

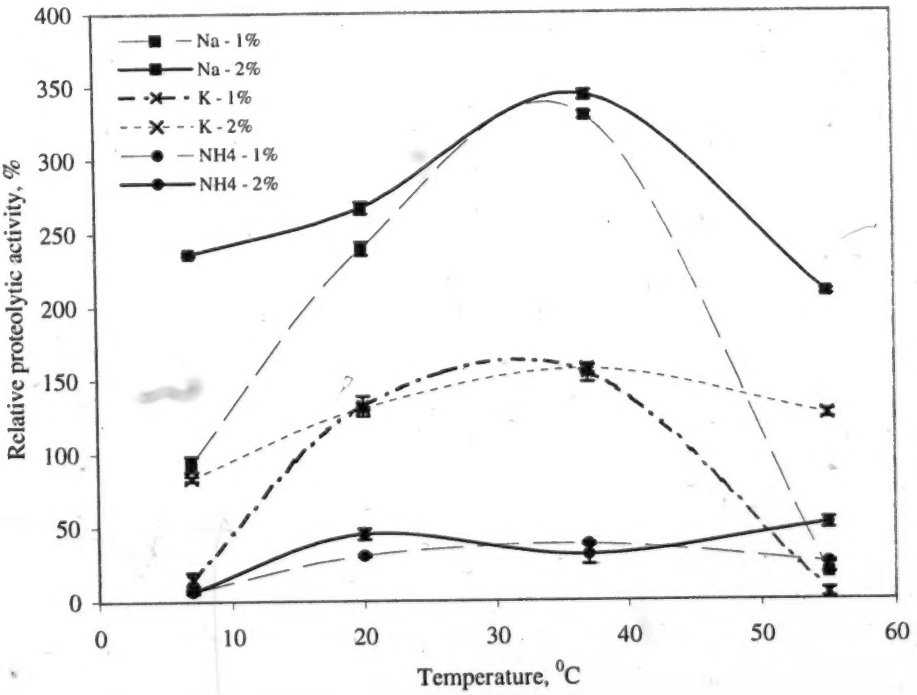

Figure 4-5: The relative proteolytic activity of the CCEs produced with NaOH, KOH or NH_4OH during the lactose hydrolysis in skim milk at different temperatures with the addition of 1 or 2% of a given CCE
(bars present ± standard deviation)

4.4. Conclusions

An earlier study on the lactose hydrolysis in skim milk using the CCE approach (Kreft and Jelen, 2001) ascertained applicability in broad range of lactose concentrations at elevated temperatures. The present study showed the impact of the neutralizers, used for pH maintenance during the cultivation of *Lactobacillus bulgaricus* 11842, on the lactose hydrolytic as well as protease activities in skim milk. The overall rate of lactose hydrolysis was the highest at 55°C, while at 65°C, the highest initial rate was not

sustained as the β-galactosidase was deactivated after 1.0 - 1.5 h in all CCEs. The catalytic constant k_{cat} followed an Arrhenius plot and was unaffected by the origin of the CCE. The undisrupted cells in all CCEs grew at moderate temperatures (20 and 37°C), resulting in the pH decrease of the lactose hydrolyzed skim milk, but the cell counts were substantially reduced < 10^2 CFU mL^{-1} at 55°C.

The proteolytic activity of the Na and K CCEs was enhanced several fold at 20 or 37°C; but was negligible at 55°C for all CCEs especially with the addition at 1% (v/v) rate. The use of NH_4OH as the neutralizing agent produced the CCEs, with the highest lactose hydrolyzing activity; however, its effect on the sensory properties of the hydrolyzed milk upon cursory tasting appeared to be grossly detrimental. A thorough evaluation of the sensory characteristics introduced by the CCE approach is necessary.

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Chapter 5*

Exopolysaccharide Formation and Proteolysis during Cultivation of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 for β-Galactosidase Production

5.1. Introduction

Lactose intolerance, the abundance of whey resulting from increased cheese making activities and some technological problems associated with lactose crystallization in the production of concentrated dairy products are the main reasons why lactose hydrolysis has been attracting the interest of scientific community for decades (Holsinger, 1978; Gekas and Lopez-Leiva, 1985; Patocka and Jelen, 1988; Zadow, 1993; Lee and Krasinsky, 1998). Although different potentially industrially applicable approaches to lactose hydrolysis have been tested, such as acid hydrolysis, the use of β-galactosidase (E.C. 3.2.1.23) remains the only method applicable in the dairy industry due to the enzyme activity profile at moderate temperatures and pH ranges.

Lactic acid bacteria (LAB), especially thermophilic species of the mixed yogurt culture, such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 (LB 11842), have been identified as high β-galactosidase producers (Itoh et al., 1980; Rao and Dutta, 1981; Greenberg and Mahoney, 1982; Shah and Jelen, 1990). The selection and optimization of a medium for the maximum β-galactosidase production by LB 11842 has been a subject of several recent reports (Bury et al., 1998;

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* A version of this chapter has been submitted for publication: Vasiljevic, T. and P. Jelen (2002). *International Dairy Journal.*

Bury at al., 2000; Chapter 3, this thesis). The addition of essential nutrients was necessary to enhance the growth and enzyme production of the culture, especially when using whey-based media. Despite media enrichment, the process for the production of β-gal-containing crude cellular extract from LAB culture was considered potentially feasible and worthy further investigation (Bury and Jelen, 2000). The cost of the medium handling after fermentation was however disregarded in the study. On the other hand, Vasiljevic and Jelen (2001) confirmed the superior growth and β-galactosidase activity of LB 11842 produced in skim milk in comparison to other whey-based media.

Although enrichment of skim milk with peptides or amino acids may result in a better growth and β-galactosidase activity enhancement, it also might cause undesirable carry over of off-flavors either in the reprocessed fermentation medium or lactose hydrolyzed products obtained by the action of the crude enzymatic extract. Therefore, the optimization of the environmental conditions, without enrichment, would be preferable for the maximization of growth parameters.

LB 11842 grows well in milk due to a well developed proteolytic system. Growth is further enhanced if skim milk is pretreated with proteases or supplemented with amino acids or peptides (Thomas and Pritchard, 1987; Garcia-Garibay and Marshall, 1991; Abraham et al., 1993). This strain preferentially utilizes lactose even in the presence of glucose because of a rather inefficient glucose import system (Chervaux et al., 2001). Lactose, imported into the cell by a secondary transport system, is cleaved by intracellular β-galactosidase. Glucose is further converted through glycolysis to lactic acid, while galactose is excreted into the medium. Besides nutritional factors, the growth and β-galactosidase activity of the culture are also affected by the environmental

conditions such as pH, temperature and oxygen tension. The optimum pH for the growth and maximum intracellular β-galactosidase activity of *Lb. bulgaricus* is 5.8 (Beal et al., 1989; Venkatesh, 1998). The pH control requires the addition of a neutralizer to counteract the lactic acid formation. Lactates possess antimicrobial properties, above that what would be expected from their water activity lowering effect (de Wit and Rombouts, 1990).

The addition of a counter ion, such as Na^+ or K^+, might further affect the growth and β-galactosidase activity of the culture and indirectly influence the properties of the crude enzymatic extract. The reports on effects of neutralizing agents on the culture properties are rather limited, although either NaOH or NH_4OH are frequently used for the pH maintenance during fermentation such as in the preparation of cheese starter cultures.

The objective of this study was to examine the impact of three different neutralizers (NaOH, KOH, NH_4OH) on the growth characteristics and enzymatic (proteolysis and β-galactosidase) activities of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, cultivated in skim milk at constant pH and to study the properties of the spent fermentation medium.

5.2. Materials and Methods

5.2.1. Culture preparation and fermentations

Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842 (LB 11842) was obtained from the University of Alberta, Department of Agricultural, Food and Nutritional Science. To prepare the inoculum for the experimental fermentation trial, 150 mL of sterile skim milk was inoculated by 1 mL of the culture and incubated at 43±1°C and

150±25 RPM in a controlled environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ) for 10 hours. Then, 50 mL of this inoculum was added into 1.5 L of fermentation medium, prepared from commercial fluid skim milk (DairyWorld Foods, Vancouver, BC) by sterilization at 121°C for 15 min and placed in a 2 L fermentor (New Brunswick). The temperature was controlled by holding the fermentor in a water bath at 43±0.2°C. The pH was maintained at pH 5.6±0.1 by the automatic addition of a 2M hydroxide (ammonium, sodium or potassium) via a peristaltic pump (Watson-Marlow, Cornwall, UK) controlled by a pH controller (Omega Engineering Inc., Stanford, CO). During fermentations, the amount of total lactic acid production was determined from the amount of a given hydroxide added. The rate of the culture growth in the fermentation medium was assessed by determining viable cell counts every two hours from the beginning of fermentation. Viable cells were enumerated as colony forming units (CFU) mL^{-1} by surface plating dilutions on the MRS (de Man et al., 1960) medium (55 g L^{-1}, Difco, Detroit, MI) with agar (15 g L^{-1}, Difco) and incubated under aerobic conditions at 43°C for 48 hours. Fermentations were performed aerobically and terminated after 10 hours. All trials were replicated three times.

5.2.2 Determination of β-galactosidase activity, proteolysis and sugar utilization patterns

The β-galactosidase activity was determined by cryoscopy, while the ONPG (*o*-nitrophenyl-β-D-galactopyranoside) method (Vasiljevic and Jelen, 2001), was also used as a confirmatory method. For the cryoscopic determination of β-galactosidase activity of the culture during growth in skim milk, 5 mL of the culture was sampled every two hours

from the fermentation media and centrifuged (Beckman Model J2-21, Beckman Coulter Inc, Fullerton, CA) at 4°C and 4500 $\times g$ for 15 min for the biomass collection. Unwashed cells were resuspended in the 5% (w/v) lactose solution in 0.1 M phosphate buffer solution (Fisher brand, pH 6.8). Under cooling in an ice-water bath to prevent the loss of activity (Shah and Jelen, 1991), resuspended cells were sonicated using the intermediate tip of a Sonic 300 dismembrator (Artek Systems Corporation, Farmingdale, NY) at 60% intensity for 4 min. Immediately after sonication, samples were placed into a water bath and lactose hydrolysis was monitored at 55°C for 30 min. Theoretically, the complete hydrolysis of a 5% lactose solution would produce the freezing point depression of 274 mH (Hortvet) (Jeon and Bassette, 1982). Freezing point measurements were performed with an Advanced Cryomatic Milk Cryoscope (Advanced Instruments Inc., Norwood, MA), model 4C2. The degree of hydrolysis was calculated as reported before (Vasiljevic and Jelen, 2002) and results expressed as activity units (U) per mL of culture. A unit of the β-galactosidase activity was defined as μmol of lactose hydrolyzed per min under the conditions described above. All measurements were at least replicated.

5.2.3. Culture proteolytic activity

The effect of the three employed neutralizers on the magnitude of the proteolytic activity of the growing culture in the fermentation media was assessed by peptide mapping using an HPLC method. A 1 mL sample was drawn every 2 h from the cell-free supernatant that remained after the centrifugation of the fermentation media for the β-galactosidase activity determination (see above). The sample, diluted with deionized water (1:4), was filtered through a 0.22 μm Millipore membrane and injected in a reverse

phase HPLC column (Sephasil peptide C18 5μ ST 4.6/100 column, Amersham Biosciences Corp, Piscataway, NJ), incorporated in a Shimadzu Ezchrom Chromatography processing system (Shimadzu precision instruments, Torrance, CA). The peptides were eluted at room temperature (approx 20°C) using a gradient of two mobile phases consisting of trifluoroacetic acid and acetonitrile. Both agents were prepared in deionized water. The eluted peptides were detected using an UV/VIS detector (Shimadzu SPD-10A) operating at 220 nm. The concentration of acetonitrile was increased linearly from 0 to 60% during a 20 min HPLC run. The total flow rate of solvents was set at 1 mL min^{-1}. The relative proteolytic activity (Rpa, %) of the culture during the growth in skim milk was inferred from the change in the total surface area under the detected peaks and calculated from the following equation:

$$Rpa,\% = \frac{TPE_e - TPE_b}{TPE_b} \cdot 100 \qquad \text{Formula 5.1.}$$

where: TPE_e and TPE_b are total peak area at the end and beginning of hydrolysis, respectively.

The change in the lactose, glucose and galactose concentrations in the cultivation media during the culture growth was determined using a carbohydrate HPLC column (Aminex HPX-87H, Biorad, Hercules, CA) in accordance with the previous report (Vasiljevic and Jelen, 2002). Briefly, the sugars were purified by alcohol extraction, vacuum-dried and resuspended in deionized water. The individual sugars, isocraticly eluted by 0.005 M H_2SO_4 at 35°C and 0.6 mL min^{-1} flow rate, were detected by a

refractometer. All determinations were performed in duplicate and the sugar concentrations, g L^{-1}, were ascertained from corresponding calibration curves.

5.2.4. Water activity (a_w) and viscosity determinations of fermentation medium

The water activity (a_w) of the fermentation medium was determined at the beginning and the end of the fermentation. Approximately 10 mL of a sample was placed in a plastic cup and loaded into an osmometer (Aqua Lab CX-2, Decagon Devices, Inc., Pullman, WA). The reading was taken after equilibration. All measurements were performed in triplicate.

Immediately after termination of the fermentation, the medium was centrifuged (Beckman model J2-21, Beckman Coulter Inc, Fullerton, CA) at 4°C and 4500 *x g* for 10 min for the cell separation. The supernatant, referred to as spent skim milk (SSM) was collected and processed using ultrafiltration (UF) to a volume concentration ratio (VCR) of 3. The SSM was concentrated on a pilot-scale RO-UF apparatus DDS LAB-20 (De Danske Sukkerfabrikker, Nakskov, Denmark) described by Tarnawski and Jelen (1985), with ten sandwiches and the total effective filtration area of 0.36 m^2. The GR60PP (polysulfone-polypropylene) UF membrane, with the nominal molecular weight cut-off of 25 kDa was supplied by Union Filtration (Nakskov, Denmark). A 2 L batch of SSM was circulated from an insulated feed tank of 20 L capacity through the module with a high pressure piston pump (model 16.50, Rannie, Copenhagen) at 10°C (±1), at 10 bars and a flow rate of 6 L min^{-1}.

Viscosity of the selected retentates (ammonium and sodium) were determined by the consecutive fixed speed test using a PAAR Physica UDS 200 rheometer (Glenn

Allen, VA) equipped with a Peltier heating system (Burkus, 2001). Tests were performed at 20±0.03°C using the DG 27 cup and bob geometry with double gap and a nominal 7 mL sample size. The actual sample size was not measured by volume due to difficulties with highly viscous samples. Instead, the clean DG 27 cup was placed on the balance, tared and 7.20±0.01 g of sample was weighed directly into the cup. The rheological properties of the retentates were also characterized by describing their rheological behavior using the power law model (Mun et al., 1999):

$$S = c \cdot R^n$$ Formula 5.2.

where c is the consistency coefficient, and n the flow behavior index. S and R represent shear stress, Nm^{-2}, and shear rate, s^{-1}, respectively.

5.3.5. Exopolysaccharide purification and monosaccharide content analysis

The observation of the high viscosity of ammonium-produced retentates, as well as the slimy appearance of the corresponding cell paste and the sugar utilization profile observed, prompted further investigations. For comparison, only the Na-produced cell-free fermentation medium was included in the examinations as the Na- and K- retentates were very similar. The non-Newtonian behavior of fluids is usually caused by hydrocolloids; in our study, an assumption was made that the culture produced exopolysaccharides. The isolation of exopolysaccharides was carried out using a 500 mL of the cell free media after fermentation. The milk proteins were precipitated by the addition of equal volume of 20% trichloroacetic acid and removed by centrifugation

(15,000 *x g*, 30 min, 4°C). Three volumes of cold absolute ethanol were then added for the overnight precipitation of polysaccharides and collected by centrifugation (De Vuyst et al., 1998). The collected pellet was redissolved in demineralized water and the whole procedure was repeated for the removal of the residual proteinaceous material. The obtained pellets were dried at 35°C in a vacuum oven and weighed. The results are expressed as the exopolysaccharide dry matter (EDM) L^{-1} culture medium

For the preliminary determination of the EPS monosaccharide composition, acid hydrolysis was carried out by redisolving dried EPS samples in 2 M HCl and incubating for 2 h at 100°C. After the hydrolysis, the solutions were neutralized with 2 M NaOH and the monosaccharide composition was determined by HPLC, using the setup as described above; the relative ratio of the peak areas was calculated to estimate the composition.

5.2.6. Statistical analysis

All fermentations were performed in triplicate and all subsequent analyses were carried out at least in duplicate. The statistical analysis of all available data was performed using the General Linear Model of SAS (SAS Institute, 1992) and a Tukey's test was used to separate the variability of means. Statistical significance was considered at $\alpha = 0.05$. The results are presented as means (n= 6 or more) ± adjusted standard error of the mean (SEM).

5.3. Results and discussion

5.3.1. Effect of neutralizers on culture growth, lactic acid production and β-galactosidase activity

The LB 11842 is a fastidious microorganism, requiring numerous nutrients for enhancement of growth and β-galactosidase activity (Bury et al., 2000; Vasiljevic and Jelen, 2001). While the effects of media composition, pH and temperature on the growth characteristics of lactobacilli have been explored extensively, little is known about the effect of neutralizers used for the pH maintenance. The investigation of such effects was one of the primary objectives of this study and the results are presented in Table 5-1.

The effects of sodium or potassium hydroxide on all growth parameters were comparable. In contrast, the use of ammonium hydroxide resulted in significantly ($p<0.05$) higher cell counts, total lactic acid production and β-galactosidase activity, reaching the values of 9.32 (±0.02) log CFU mL^{-1}, 117.0 (±2.09) mL of 2 M hydroxide L^{-1} media and 335.2 (±0.05) U mL^{-1} culture, respectively, at harvesting. The cell counts acquired in this study were similar to those reported previously (Vasiljevic and Jelen, 2002). Although the β-galactosidase activity of the cultures resulting from the use of the three neutralizing agents were significantly different ($p<0.05$), the difference between the specific β-galactosidase activities (U CFU^{-1}) was not significant ($p=0.0564$). This was determined using Tukey's multicomparison test for the preset significance level, $\alpha=0.05$ (Table 5-1). The β-galactosidase activity of the growing culture was measured by cryoscopy (Table 5-1), while the ONPG test, used as a confirmatory method, showed a similar pattern for all fermentations (Appendix, Figure A-3 and A-4).

The presence of higher concentrations of sodium or potassium may negatively affect the culture growth due to osmotic stress (Hefnawy and Marth, 1993). However, the growth rate of bacteria in media containing NaCl or other salts is mainly a function of water activity (a_w) and generally not of the concentration of a particular solute or solutes (Scott, 1957; Wodzinsky and Frazier, 1960). Besides having a significant ($p<0.05$) effect on all growth characteristics in comparison to the other two neutralizers, the addition of ammonium hydroxide also resulted in a significantly ($p<0.05$) higher growth rate (0.50 ± 0.02 h^{-1}) than when sodium or potassium hydroxide was used (0.43 ± 0.02 and 0.42 ± 0.02 h^{-1}, respectively).

Table 5-1: The cell count, lactic acid production and β-galactosidase activity at harvesting of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 cultivated in skim milk for 10 hrs at pH 5.6 maintained by NaOH, KOH or NH_4OH

Neutralizer	Cell count, log CFU mL^{-1}	Lactic acid, cumul. neutr. mL L^{-1} media	β-gal activity, U mL^{-1}	Specific β-gal activity, U$x10^{-7}$ CFU^{-1}
NaOH	9.16^a	93.6^a	273.5^a	1.89^a
KOH	9.04^a	93.5^a	262.0^a	2.41^a
NH_4OH	9.32^b	117.0^b	335.2^b	1.62^a
SEM	0.028	2.09	5.18	0.136
R^2	0.94	0.96	0.97	0.85

(Means in columns with different letters are significantly different, $\alpha=0.05$; SEM – adjusted standard error of the mean; R^2 – coefficient of determination)

The final concentrations of sodium and potassium ions in the fermentation media were below 0.4% w/v, which in turn may have positively affected the culture growth (Gow et al., 1981). The effect of all cations on the water activity was similar and no significant differences ($p>0.05$) were observed (Appendix, Table A-2), indicating that the

effect of the water activity of the media on the growth characteristics of the culture was not a factor causing the differences between the growth rates. Lactic acid also may negatively influence the culture growth during the batch fermentation, affecting pH homeostasis through a mechanism that is not solely dependent upon the non-dissociated acid molecule or H^+ concentration (Goncalves et al., 1997). The cell counts during ammonium hydroxide fermentations reached the maximum at harvesting even though the lactic acid production was significantly ($p<0.05$) higher in comparison to other two neutralizers (Table 5-1). The maximum cell counts for sodium- and potassium-controlled fermentations were achieved at 8 h, reaching values of 9.18 and 9.10 (±0.05) log CFU mL^{-1}, respectively. The cell counts for these two neutralizers declined slightly at the end of cultivation, indicating that ammonium ion might have a protective effect on the growing cells.

5.3.2. Effect of neutralizers on the proteolytic activity of the culture

The peptide mapping of the cell-free spent fermentation media was used to estimate the proteolytic activity of the culture during the fermentations (Figure 5-1). While there were no significant ($p>0.05$) differences in the proteolytic activity among the three neutralizers after two h, major differences were noted after 4 h. The addition of neutralizers started immediately after the media pH reached 5.6, approximately 2 h after commencing fermentation in all trials. The culture exerted significantly ($p<0.05$) lower proteolytic activity with ammonium hydroxide (120±2.45%) in comparison to other two neutralizers (174.4 and 161.4±2.45% for NaOH and KOH, respectively) as ascertained at the end of fermentations. Apparently, LB 11842 was somehow able to utilize ammonium

in energy conservation that was redirected towards the enhancement of all growth characteristics. This observation also illustrates the reason for the lower proteolytic activity of the β-galactosidase-containing CCE produced from the ammonium hydroxide fermentation in comparison to the two other neutralizers observed during the lactose hydrolysis trials in skim milk (Chapter 4, this thesis).

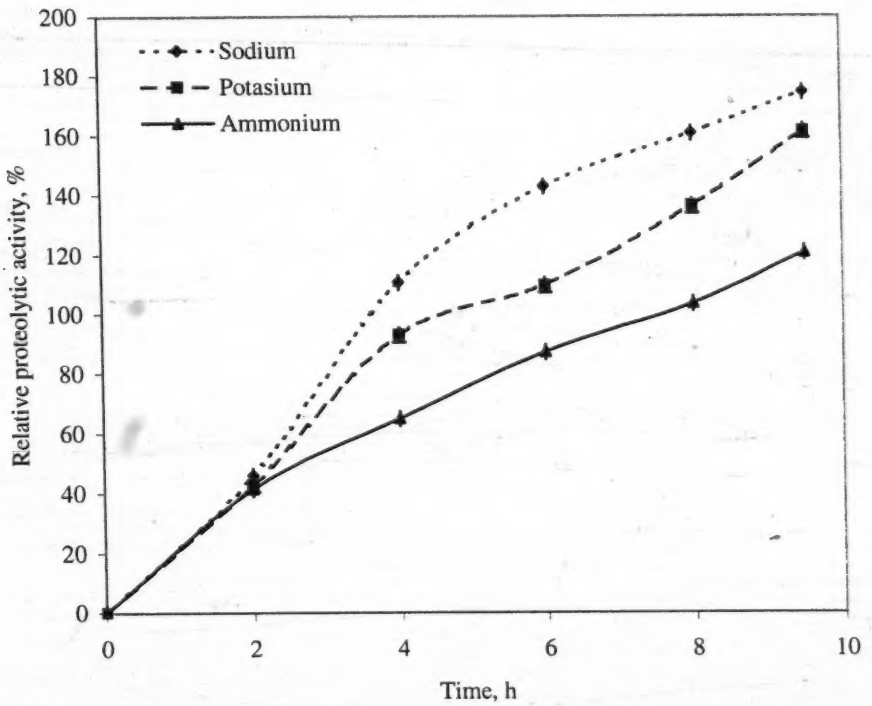

Figure 5-1: Relative proteolytic activity (%) of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 during cultivation in skim milk at 42°C and pH 5.6 maintained by NaOH, KOH or NH_4OH as neutralizers
(bars present adjusted standard error of the mean, ±2.45%)

Lactobacillus delbrueckii ssp. *bulgaricus* is an auxotrophic organism, requiring multiple essential amino acids for growth. In the absence of available free amino acids as

in milk, it relies on its own proteolytic system to fulfill the necessary requirements for the nitrogen source (Cheraux et al., 2001). The effect of ammonium on the growth parameters of *Lb. delbrueckii* subsp. *bulgaricus* strains still remains obscure. Murad (1998), using the addition of ammonium phosphate to a whey permeate medium, achieved several fold increase in the β-gal activity. No other growth parameters were monitored. Zayed and Winter (1995) slightly improved the growth and lactic acid production of several unidentified *Lactobacillus* strains after fortification of salted whey with yeast extract, ammonium sulfate and potassium orthophosphate. On the contrary, ammonium showed no effect on growth of selected *Lactobacillus* strains cultivated in a chemically modified medium (Cheraux et al., 2001). Furthermore, the presence of readily available peptides in the fermentation medium would suppress the proteolytic activity of the cell-envelope associated proteases (Hebert et al., 2000).

5.3.3. The lactose utilization profile and media viscosity as affected by the neutralizers

Lb. delbrueckii ssp. *bulgaricus* utilizes the glucose moiety of lactose, while galactose is excreted into the medium in a concentration practically equimolar with that of the utilized lactose. The lactose metabolism of *Lb. bulgaricus* 11842 followed the expected pattern during the fermentation with sodium or potassium hydroxide (Figure 5-2). However, with ammonium hydroxide, the galactose concentration in the medium remained lower than predicted until the end of fermentation. At the same time, utilization of lactose was more extensive after about 2.5 h of fermentation. Furthermore, the viscosity analysis of the cell-free retentates obtained by ultrafiltration from the NaOH- or NH_4OH-fermentation media to VCF 3, gave significantly different ($p<0.05$) results

(Figure 5-3). In contrast to the retentate produced with NaOH, the viscosity of the NH_4OH retentate was substantially higher than that of 5X concentrated skim milk retentate determined under similar conditions (Mun et al., 1999).

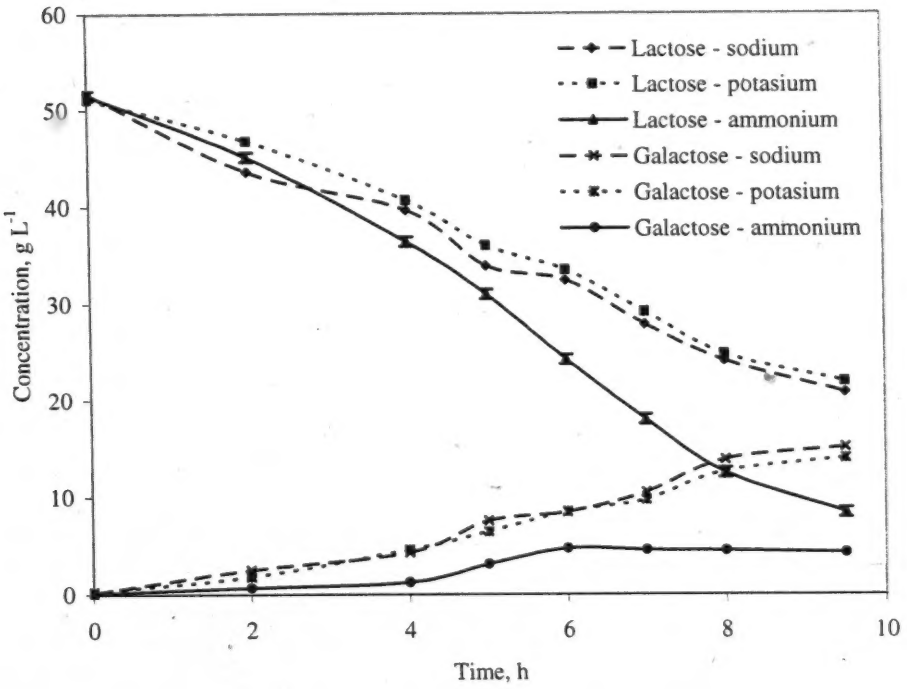

Figure 5-2: Lactose utilization and galactose accumulation in the fermentation media during the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 in skim milk at 42°C and pH 5.6 maintained by NaOH, KOH or NH_4OH as neutralizers
(bars present adjusted standard error, ±0.494 and 0.131 g L^{-1} for lactose and galactose, respectively).

The rheological properties of the investigated retentates were further examined by power law model fitting and the obtained parameters are presented in Table 5-2. The obtained results clearly show that retentates produced with NH_4OH had non-Newtonian behavior and apparent pseudoplasticity. Also this retentate demonstrated a significant

shear thinning behavior as the apparent viscosity decreased almost 6 fold as the shear rate increased. In contrast, retentate produced with NaOH had a flow behavior index, n, approaching 1 indicating Newtonian behavior.

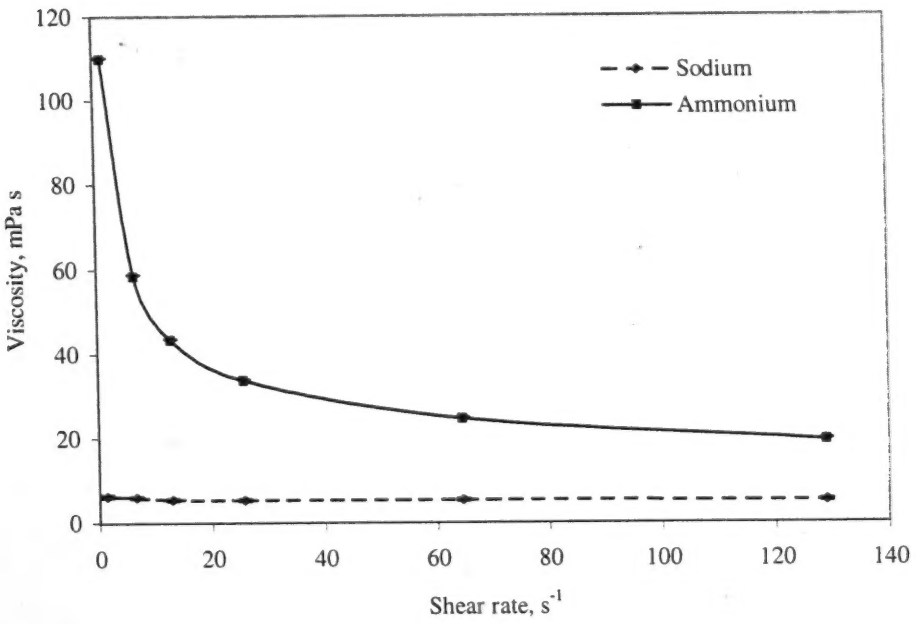

Figure 5-3: Viscosity of UF retentates (VCR 3) from the NaOH- or NH_4OH-produced spent skim milk
(bars present adjusted standard error, ±0.29 mPa s)

The effect of KOH on the rheological properties of spent fermentation media retentate was not investigated because of similarities with media produced with NaOH in all monitored and visually observed parameters. The increase in viscosity during the concentration of skim milk is caused mainly by protein/protein interaction. Both retentates in our study underwent a similar treatment (the difference being the kind of neutralizer use in the fermentation) and contained a similar concentration of proteins. The

low viscosity of the NaOH retentate might have also been affected by higher proteolytic activity of the culture. The much higher viscosity of the NH_4OH – produced retentates, however, was likely caused by a presence of other compounds, presumably exopolysaccharides rather than by protein-protein interactions, which presumably would be the same as when NaOH was used.

Table 5-2: Power law model parameters of retentates produced by ultrafiltration to VCF 3 from the NaOH- or NH_4OH-produced spent skim milk

Neutralizer	c, mPa s	n
Ammonium	0.118^a	0.622^a
Sodium	0.006^b	0.956^b
SEM	0.3×10^{-3}	2.5×10^{-3}
R^2	0.999	0.998

(Means in columns with different letters are significantly different, α=0.05; SEM – adjusted standard error of the mean; R^2 – coefficient of determination)

5.3.4. Exopolysaccharide production and composition

LB 11842 has not been identified as an EPS producer so far. As presented in Figure 5-2, the galactose concentration in the medium increased during fermentation with NaOH or KOH in contrast to a decrease during NH_4OH fermentation, presumably due to conversion into exopolysaccharides as additionally indicated by the viscosity measurements (Figure 5-3). The EPS production by LAB is generally influenced by media composition, carbon/nitrogen ratio and environmental conditions (Degeest and De Vuyst, 1999). As determined in our study, LB 11842 produced exopolysaccharides

regardless of the neutralizer used during cultivation. However, the polymer production was almost 3 times higher in the presence of ammonium than sodium (428.6 and 154.7±15.1 mg EDM L^{-1}, respectively). This may be an indication that the EPS production might have been induced in the presence of ammonium by changing the carbon/nitrogen ratio or by enabling the transcription of the chromosomally encoded *eps* genes. Gassem et al. (1997) showed that the addition of casamino acids and NH_4Cl increased the EPS production of a *Lb. delbrueckii* subsp. *bulgaricus* strain. Also, nonropy strains of *Lb. delbrueckii* subsp. *bulgaricus* produced larger amounts of capsular EPS if cultivated in milk and some of them formed capsules only in the presence of lactose but not glucose (Hassan et al., 2001).

The preliminary compositional analysis of the exopolysaccharides, acquired by HPLC analysis, resulted in approximately 1:5 glucose/galactose ratio for both fermentations. Approximately 5% of the EPS monosaccharides remained unidentified, although the polymer composition and content was in a broad agreement with results reported for EPS produced by *Streptococcus thermophilus* (De Vuyst et al., 1998). Van Geel-Schutten et al. (1998) also reported that the EPS, produced by different *Lactobacillus* strains cultivated in chemically defined media containing lactose as a sole carbon source, were composed of mainly mannose, galactose and glucuronic acid. The galactose content was between 20 and 25%. The EPS produced by LB 11842 in our study showed a poor solubility upon reconstitution, which improved at low pH, suggesting the presence of nucleophilic groups, possible amines. Several studies (Macura and Townsley, 1984; Garcia-Garibay and Marshall, 1991), investigating the EPS production by *Streptococcus* and *Lb. delbrueckii* subsp. *bulgaricus* strains, reported similar findings and

the conducted compositional analysis revealed that the EPS were actually glycoproteins of different protein/carbohydrate ratio.

The ammonium-based cell paste produced in our study was distinctly different (visually observed sliminess) from that obtained with either NaOH or KOH. This might indicate that the EPS produced were either capsular or more tightly bound to the cell wall. Further analysis may be required especially since some of the galactose was unaccounted for (Figure 5-2 and determined EPS content in the fermentation medium after termination). Looijesteijn and Hugenholtz (1999) observed no change in EPS production by *Lactococcus lactis* subsp. *cremoris* when sodium hydroxide was replaced by ammonium hydroxide, although the EPS association with the cell wall increased. In contrast, our results show that NH_4OH had a major influence on the EPS production by *Lb. bulgaricus* 11842. This fact may be of a special interest to the dairy industry because *Lb. delbrueckii* subsp. *bulgaricus* is used in the yogurt production as a part of the starter culture. The enhanced EPS production by the culture, if induced by the presence of ammonium ion or possibly by other means, may result in natural thickening, improving the texture and stability of yogurt and preventing syneresis.

5.4. Conclusions

Lb. delbrueckii ssp. *bulgaricus* 11842 produces exopolysaccharides in controlled pH fermentations using skim milk. The amounts of EPS produced were much higher with NH_4OH in comparison to NaOH as a neutralizer. However, the EPS from both fermentations had putatively similar composition. The higher EPS production - and possibly different EPS structure - resulted in highly pseudoplastic behavior of NH_4OH–

produced UF retentates. The use of NH_4OH also resulted in enhanced growth, higher lactic acid production and increased β-galactosidase activity of the culture in comparison to the other two neutralizers; however, the higher β-galactosidase activity was due to higher growth with no significant differences in the specific β-galactosidase activities. The proteolytic activity of the culture was suppressed in the presence of NH_4OH; along with the enhanced growth, this may indicate the NH_4OH utilization by the growing cells. Further research is necessary to define the modes of ammonium utilization and of the EPS production by *Lb. bulgaricus* 11842 and to explore the effects of EPS presence on the downstream processing of the crude cellular extract and the cell-free fermentation media.

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Chapter 6*

Oligosaccharide Production and Proteolysis during Lactose Hydrolysis Using Crude Cellular Extracts from Lactic Acid Bacteria

6.1. Introduction

The lactose hydrolyzing ability of crude cellular extracts (CCE) containing β-galactosidase (EC 3.2.1.23, β-gal) from mechanically disrupted *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (Lb11842) has been explored extensively (Shah and Jelen, 1991; Jelen, 1993; Kreft and Jelen, 2000; Kreft et al., 2001; Chapter 4, this thesis). However, little attention has been paid to date to catalytic ability of the CCE regarding the transferase reactions, another important property of bacterial β-gal. The proteolytic activity of the Lb11842 CCE preparation was described in a preliminary way (Vasiljevic and Jelen, 2002a). Such activity, if not controlled properly, could interfere with the lactose hydrolysis. Alternatively, for some uses of the CCE, the proteolytic reactions may be desirable and thus these should be well characterized.

The transferase reactions include the internal rearrangement of the lactose molecule with the formation of different disaccharides, as well as transgalactosyl reactions, resulting in creation of tri- and higher oligosaccharides (Prenosil et al., 1987a). The transferase products are hydrolyzed very slowly by the human β-galactosidase in the small intestine, which may result in the gastrointestinal discomfort and lactose intolerance-like symptoms (Mahoney, 1998). More recently, several studies showed the

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* A version of this chapter has been submitted for publication: Vasiljevic, T. and P. Jelen (2002). *Le Lait*.

positive effect of the oligosaccharide addition on the microbial microflora, influencing the increase of fecal *Bifidobacteria* and modifying the colonic fermentation metabolism in the gut of healthy humans (Bouhnik et al., 1997), or reducing the colon cancer risk in carcinogen-treated rats (Gallaher & Khil, 1999).

The transferase reactions have been described for highly purified β-gal preparations obtained from a number of microbial sources (Huber et al., 1976; Toba and Adachi, 1978; Toba et al., 1981, 1985; Mozaffar et al. 1985; Smart, 1991; Garman et al., 1996). Using β-gal in the batch mode was suggested for maximization of the oligosaccharide production (Prenosil et al., 1987b). No attempt has been made so far to study the oligosaccharide producing capability of the β-gal-containing CCE, although the work of Kreft et al. (2001) offered some indications that the oligosaccharides were indeed produced during lactose hydrolysis by Lb11842 CCE in buffered lactose solutions.

Thermophilic LAB, especially mixed yogurt cultures, possess appreciable proteolytic activity (Shihata and Shah, 2000). The presence of cell-envelope proteases and different intracellular peptidases result in an efficient breakdown of casein, major milk protein, into different amino acids and peptides required for the cell growth (Tsakalidou et al., 1999). Several studies reported pH and temperature dependence of the protease activity of several thermophilic LAB species (Abraham et al., 1993; Fira et al., 2000). The high proteolytic activity of a β-gal preparation could be undesirable during the lactose hydrolysis in milk since it may result in bitterness (Modler et al., 1993). Proteolytic activity in CCE preparations would be expected, since CCE are mixtures of liberated intracellular enzymes as well as cell debris. The minimization or maximization of the proteolytic activity by controlling the environmental conditions (temperature, time,

substrate concentration) during the lactose hydrolysis may be therefore required depending on a targeted use of the β-gal-containing CCE.

The main objectives of our study were to 1) compare the lactose hydrolyzing ability of the β-gal-containing CCEs prepared using three alternative thermophilic LAB sources in buffered lactose and skim milk systems at different temperatures; 2) characterize the transferase reactions during the lactose hydrolysis in buffered lactose and skim milk systems at different temperatures by the three β-gal-containing CCE preparations; and 3) describe proteolytic activity of the three CCE preparations during the lactose hydrolysis in skim milk. The well-characterized *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 was included in these studies for comparison to *Lactobacillus delbrueckii* subsp. *lactis* DMF 3078 and *Streptococcus thermophilus* 143, two other organisms with known high β-gal producing capability.

6.2. Materials and Methods

6.2.1 Culture cultivation and cell collection

Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842 (Lb11842) was obtained from University of Alberta, Department of Agricultural, Food and Nutritional Science. *Lactobacillus delbrueckii* ssp. *lactis* DMF 3078 (Lb3078) and *Streptococcus thermophilus* 143 (St143) were provided by the Department of Milk and Fat Technology, Institute of Chemical Technology, Prague, Czech Republic. The cultures were cultivated in pasteurized commercial fluid skim milk (Lucerne milk processing plant, Edmonton, AB) as described by Geciova et al. (2002). Fermentations were conducted for 10 h in a 50 L fermentor at 43°C and pH, maintained by 10 M KOH, set at 5.6 for *Lactobacillus* sp.

and 5.8 for *Streptococcus*. After fermentation, the cell collection and handling were performed as reported previously (Vasiljevic and Jelen, 2002b). Before the production of the CCE, the cell paste samples from each fermentation were randomly selected for the determination of the total solids and the β-gal activity by oven drying and the ONPG (o-nitrophenyl-ß-D-galactopyranoside) method, respectively (Chapter 3, this thesis). A unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyze 1 μmol of ONPG min^{-1} at 37°C under conditions described previously (Chapter 4, this thesis).

6.2.2. Preparation of crude cellular extracts and reaction mixtures

The crude β-galactosidase preparations were obtained similarly as described before (Chapter 7, this thesis). Prior to cell disruption for the liberation of the intracellular β-gal, the frozen cell paste (14 to 16% dry matter) was thawed at 37°C in a water bath and reconstituted either in pH 6.8 skim milk salt buffer - SMSB (Santos et al., 1998) or a 10% (w/v) solution of reconstituted low-heat skim milk powder (Dairyworld Foods, Vancouver, BC) yielding cell paste preparations containing approximately 10% total solids in both cases. The liberation of intracellular β-gal was achieved using a one-pass treatment with two ceramic disrupting chambers of a microfluidizer (Model M-110EH, Microfluidics, Newton, MA) as described by Geciova et al. (2002). These treated preparations, denoted as crude cellular extracts (CCE) in further text, were used without further purification.

6.2.3. Lactose hydrolysis and quantification of reaction products

The reaction systems for the lactose hydrolysis were prepared either by dissolving appropriate amounts of lactose monohydrate (Fisher Scientific Limited, Nepean, ON, CA) in skim milk salt buffer to give 5, 12.5, 20 or 30% (w/w) final lactose concentration, or by reconstituting the low-temperature skim milk powder (Dairyworld Foods) in deionized water to prepare solutions giving 10, 20 or 30% (w/w) final total skim milk solids content upon recombining with the CCE preparations.

The CCE preparations were combined with the lactose or skim milk solutions, giving reaction mixtures containing approximately 10 U mL^{-1} β-gal activity, which corresponded to the final cell paste total solids content ranging between 2 and 3% (w/v). The reaction mixtures were held at 30, 40, 50, or 60°C for 120 min. Samples (1 mL) were taken after 30, 60 and 120 min and the reaction was terminated by alcohol precipitation (Chapter 4, this thesis). The time frame selected was based on the report of Greenberg and Mahoney (1983) indicating that maximum of oligosaccharide production in milk was achieved within 2 hrs of the lactose hydrolysis process.

The production of monosaccharides and formation of oligosaccharides were quantified by high performance liquid chromatography (HPLC) with Shimadzu Ezchrom Chromatography processing system (Shimadzu Precision Instruments, Torrance, CA). The mono- and disaccharides were separated by a Supelcosil LC-NH_2-5 μm column (Supelco, Bellefonte, PA; 25 cm length and 4.6 mm diameter). A DVB polyamine column (Jordi Gel, 250 mm length and 4.6 mm diameter, Bellingham, MA) was used to separate the oligosaccharides. Elution consisted of a gradient of two mobile phase solvents, namely deionized water and acetonitrile (HPLC grade) using a pattern similar to

the Supelcosil column. The concentration of acetonitrile was decreased linearly over twenty five minutes from 90 to 60%, increased to 100% at 26 min, and then decreased to 90% at the end of the determination. The total run time was 30 min. The total flow rate was constant at 1 mL min^{-1}. In all cases, a 25 µL sample was injected by a Hewlett Packard Series 1050 autosampler (HP, Mississauga, ON). The peaks were identified and concentrations determined by using external standard solutions of glucose, galactose, lactose, maltotriose, maltotetraose, maltopentaose and maltohexaose and corresponding calibration curves.

6.2.4. Proteolytic activity

The proteolytic activity of the CCE preparations during the lactose hydrolysis in skim milk reaction systems were assessed by HPLC peptide mapping of the hydrolyzed products. The conditions employed during the HPLC determinations were described previously (Chapter 4, this thesis). The samples (1 mL) were taken similarly as samples for the determination of the rate of lactose hydrolysis, diluted ten fold in deionized water and filtered through a 0.22 µm Millipore filter. The HPLC determinations were performed using a Sephasil reverse phase peptide column (Pharmacia Biotech) and Shimadzu Ezchrom Chromatography processing system. The change of absorbance was observed by an ultra violet/visible wavelength detector (Shimadzu SPD-10A) operating at 220 nm wavelength. Total peak area was obtained by integration of all the peaks observed. The relative proteolytic activity (RPA, %), expressing the change of the peptide profile, was calculated from the previously reported equation (Chapter 5, this thesis):

$$RPA,\% = \frac{TPE_t - TPE_O}{TPE_O} \cdot 100$$

Formula 5.1.

where: TPE_t and TPE_0 are total peak area at time t (30, 60 or 120 min) and beginning of hydrolysis, respectively.

6.2.5. Kinetic parameters

To compare the efficiency of lactose hydrolysis by the three selected β-gal containing CCE preparations in buffered lactose and skim milk systems, the kinetic parameters, Km and k_{cat}, were evaluated from the concentration of the monosaccharides released during the first 30 min of the hydrolysis for all lactose concentrations and temperatures examined. The constants were inferred from the Michaelis-Menten equation using the Lineweaver-Burk method (Whitaker, 1994):

$$\frac{dP}{dt} = \frac{k_{cat} E_o S_o}{K_m + S_o}$$

Formula 6.2.

where: dP/dt – rate of the product formation, mM min^{-1}; K_m – Michaelis-Menten constant, M; k_{cat} – catalytic constant, M $U^{-1}min^{-1}$; E_o – initial enzyme activity, U; S_o – initial substrate concentration, M.

6.2.6. Optimization study

An optimization study was performed to determine effects of three independent variables – lactose or skim milk total solids concentration (X_1), temperature (X_2) and time (X_3) – on the dependent variables, total oligosaccharides or relative proteolytic activity. All results were analyzed as a full factorial design using the response surface methodology (RSM) (Khuri and Cornell, 1996). A multivariate functionality was fitted by a second order model in the form:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j}^{k} \beta_{ij} x_i x_j + \varepsilon$$

Formula 6.2.

where Y is the predicted response (oligosaccharide concentration or relative proteolytic activity), and β_o, β_i, β_{ii}, β_{ij} the regression coefficient related to average, linear, quadratic and interaction effects, respectively. The subscript k presents number of independent variables with the i, j observation number ranging from 1 to k.

6.2.7. Statistical analysis

All experiments were at least replicated and all subsequent analyses were carried out at least in duplicate resulting in n = 4 or more. The statistical analysis using all available data was performed by the General Linear Model of SAS (SAS Institute, 1992) as a full factorial, split-plot in time design. The model included all main effects (strain, lactose concentration, temperature, time) and corresponding interactions. The covariate

analysis, using the enzyme activity or the cell paste total solids as a covariate, was employed if necessary. The statistical significance was preset at α=0.05.

6.3. Results and Discussion

6.3.1. Monosaccharide formation

Effectiveness of the CCEs from the three different LAB sources to hydrolyze lactose was compared using the determination of rate constants for the lactose hydrolysis as Km and k_{cat} values and by measuring the monosaccharide formation during the course of the lactose hydrolysis. Generally, the enzyme origin, temperature and medium in which the lactose hydrolysis was performed had significant ($p<0.01$) effect on both kinetic parameters (Table 6-1). The covariate analysis showed no significant effect ($p=0.4478$) of the initial β-gal activity in the CCEs on the kinetic parameters. Shah and Jelen (1991) and Garman et al. (1996) also reported substantial differences in the β-gal activity in several different LAB species. The Km and k_{cat} values obtained for the Lb11842 CCE in the present study were similar to those determined by a different methodology for the same CCE preparation during the lactose hydrolysis in 10% skim milk (Chapter 4, this thesis). Generally, the initial rate of the lactose hydrolysis was higher in skim milk than in the buffered lactose solutions. Mozaffar et al. (1985) reported opposite findings as they noted a substantial decrease in the initial velocity of the lactose hydrolysis by β-gal obtained from *E. coli* or *K. lactis* in milk in comparison to that in a buffered lactose solution. The product inhibition (Itoh et al., 1980), disregarded in our calculations, as well as different enzymes and/or the proteolytic activity of the CCE preparations (Chapter 4, this thesis) may likely have important ramifications on the

Table 6-1: The estimation of Michaelis-Menten type kinetic parameters, Km and k_{cat}, and the energy of activation, E_a, by Arrhenius plot for the lactose hydrolysis in lactose and skim milk preparations at different temperatures using β-gal containing CCE produced from three different thermophilic dairy cultures

CCE*/ Temperature (°C)	Lactose			Skim milk		
	Km, mM	k_{cat}, μmol U^{-1} min^{-1}	E_a, KJ mol^{-1}	Km, mM	k_{cat}, μmol U^{-1} min^{-1}	E_a, KJ mol^{-1}
Lb11842						
30	201.2	257.5		97.4	203.9	
40	244.6	294.4		198.4	293.4	
50	320.9	654.9		127.5	469.5	
60	185.3	614.0	28.68	78.8	443.2	23.71
St143						
30	575.1	272.0		28.6	124.6	
40	861.3	404.1		303.4	237.8	
50	612.4	664.3		122.9	304.0	
60	567.1	659.6	26.67	96.5	328.4	26.75
Lb3078						
30	343.5	225.0		32.0	119.6	
40	407.0	249.3		241.0	202.6	
50	1672.5	550.4		133.9	294.9	
60	431.3	411.8	23.94	94.7	300.1	26.57
SEM**	37.39	15.31	0.78	11.45	8.42	0.74
R^{2***}	0.911	0.921	0.861	0.978	0.963	0.898

***CCE – β-galactosidase-containing crude cellular extracts from Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143 – *St. thermophilus* 143, and Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; **SEM – adjusted standard error of the mean; ***R^2 – coefficient of the determination; n=12 or more.**

rate of the lactose hydrolysis. Also, the complexity and the rates of the oligosaccharide formation may substantially influence the k_{cat} and Km values (Huber et al., 1976). The temperature dependence of the catalytic constant k_{cat} followed the Arrhenius plot

resulting in significant differences ($p<0.05$) for the three LAB species studied. The energy of activation (E_a) also differed significantly ($p<0.05$) among the CCE preparations examined (Table 6-1). The values obtained in the present study were lower than those determined previously (Chapter 4, this thesis), using a different methodology.

Table 6-2: The content of monosaccharides as identified by HPLC analysis after termination of the lactose hydrolysis in buffered lactose solutions conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures

	Monosaccharides, mg mL^{-1}							
Lactose/ CCE*	Glucose				Galactose			
	30**	40	50	60	30	40	50	60
300***								
Lb11842	17.22	19.74	33.26	32.15	8	9.17	19.11	18.48
St143	15.05	15.96	24.58	20.33	7.12	7.54	14.11	10.81
Lb3078	12.59	14.73	22.35	20.08	7.33	6.68	11.25	13.08
200								
Lb11842	15.3	15.25	29.65	29.44	7.34	9.82	17.56	18.32
St143	9.99	14.09	25.61	21.72	4.37	6.48	11.05	13.82
Lb3078	8.78	12.7	17.92	17.86	5.45	5.65	10.94	12.61
125								
Lb11842	13.55	13.02	24.99	19.62	6.44	8.38	15.81	12.83
St143	10.99	13.09	23.33	15.5	6.4	6.18	11.56	10.95
Lb3078	10.13	15.32	14.59	12.8	5.54	2.63	9.77	8.54
50								
Lb11842	10.74	11.1	18.27	18.24	5.26	5.67	9.87	11.33
St143	7.5	8.2	11.99	10.56	3.52	3.98	6.17	7.32
Lb3078	7.05	7.48	8.77	9.13	3.78	4.05	4.26	5.48
SEM†	0.53				0.38			

***CCE – β-galactosidase-containing crude cellular extracts from Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143 – *St. thermophilus* 143, and Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; **temperature, °C; ***lactose concentration, mg mL^{-1}; †SEM – adjusted standard error of the mean, n = 4 or more.**

The considerable difference between rate the constants, Km and k_{cat}, describing the lactose-hydrolyzing capability of the three studied CCEs, significantly ($p<0.01$) affected the extent of the monosaccharide formation in all examined preparations. Tables

6-2 and 6-3 show the final concentrations of glucose and galactose present in lactose and skim milk solutions after the termination of the lactose hydrolysis trials. The highest degree of the lactose conversion into constitutive monosaccharides (58.7 and 62.5±0.65%, respectively) was achieved by Lb11842 CCE in 5% (w/w) lactose and 10% (w/w) skim milk preparations.

Table 6-3: The content of monosaccharides as identified by HPLC analysis after termination of the lactose hydrolysis in skim milk preparations conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures

Lactose/ CCE*	Monosaccharides, mg mL^{-1}							
	Glucose				Galactose			
	30**	40	50	60	30	40	50	60
150***								
Lb11842	13.87	18.6	27.76	29.83	7.24	9.61	17.09	16.09
St143	7.86	12.23	14.27	23.35	3.69	6.29	6.65	11.76
Lb3078	7.41	11.27	13.5	14.54	3.64	5.37	7.21	8.2
100								
Lb11842	12.3	14.01	24.39	22.35	6.89	8.17	14.07	13.19
St143	7.23	10.56	20.17	19.88	3.82	5.72	10.31	10.33
Lb3078	6.97	10.39	18.33	15.78	3.75	5.48	10.89	9.58
50								
Lb11842	14.77	11.47	19.5	18.7	8.98	7.01	11.58	11.3
St143	13.83	8.33	15.83	16.8	7.85	4.8	8.4	9.97
Lb3078	11.85	7.89	13.48	14.97	6.82	4.68	8.62	9.51
SEM+	0.26				0.34			

***CCE – β-galactosidase-containing crude cellular extracts from Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143 – *St. thermophilus* 143, and Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; **temperature, °C; ***lactose concentration, mg mL^{-1}; +SEM – adjusted standard error of the mean, n = 4 or more**

The lactose concentrations and temperatures affected the relative ratio of glucose to galactose significantly ($p<0.05$), while the enzyme origin had no apparent effect. The glucose to galactose ratio differed from the theoretical value of 1:1 and generally ranged from 1.44 to 2.32±0.10. The ratio obtained during the lactose hydrolysis in skim milk was

slightly but not significantly ($p>0.05$) lower than in lactose preparations. Similar observations about the increased glucose/galactose ratio during the early stages of the lactose hydrolysis (Huber et al., 1976, Smart, 1991) were attributed to the formation of the transferase products containing mainly galactose.

6.3.2. Transferase reactions by CCE preparations

The lactose hydrolysis by all β-gal-containing CCEs in all reaction mixtures resulted in formation of other carbohydrates in addition to the major hydrolysis products glucose and galactose. Typical chromatograms from the HPLC analysis of the lactose-hydrolyzed samples obtained using the Lb11842 CCE are shown in Figure 6-1; the use of the other two CCEs resulted in similar chromatograms (Appendix, Figure A-5 and A-6). The chromatogram "A", acquired using the Jordi oligosaccharide column, shows separation of all carbohydrates into size classes; however, separation within the classes was not achieved. A better separation for mono- and disaccharides was obtained using the Supelcosil column (chromatogram "B"), but the peak for tetrasaccharides did not appear and trisaccharides (peak 3) were partitioned poorly. The formation of tetrasaccharides in skim milk at all concentrations and temperatures was revealed only for Lb3078 CCE (Table 6-5). The reason for this observation remained unclear; either oligosaccharides formed by Lb11842 or St143 CCE were below a detectable limit or different kinetic parameters at low lactose concentrations were responsible for a kinetic shift towards the formation of monosaccharides (Huber et al., 1976). The peaks 2b and 2c appeared in all processed systems, with combined maxima up to 9.5±0.16% of the total sugars, and could likely represent disaccharides other than lactose. Huber et al. (1976) and Greenberg

and Mahoney (1983) reported formation of allolactose (6-*O*-galactosyl-β-D-glucopyranose) and 6-*O*-galactosyl-β-D-galactopyranose as major products of the transferase reactions during the lactose hydrolysis.

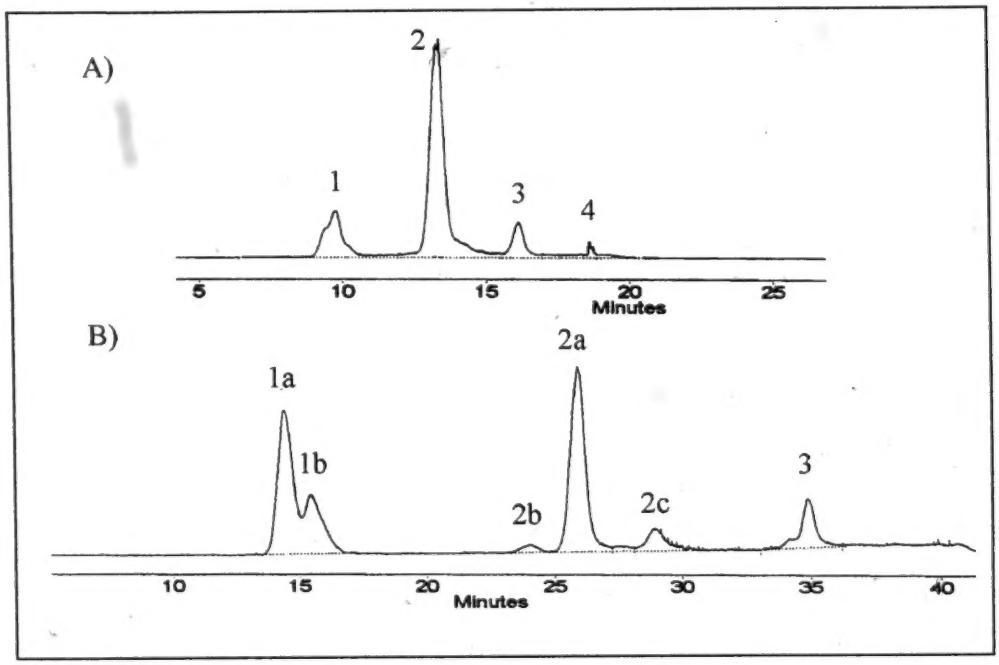

Figure 6-1: Typical HPLC chromatograms obtained analyzing products of the lactose hydrolysis by Lb11842 CCE preparation as acquired by A) Jordi oligosaccharide or B) Supelcosil carbohydrate columns
(Peaks identified as follows: 1 – monosaccharides; 2 – disaccharides; 3 – trisaccharides; 4 – tetrasaccharides; 1a – glucose; 1b – galactose; 2a – lactose; 2b and 2c – unidentified disaccharides)

The formation of pentasaccharides or hexasaccharides was not detected in any of the processed systems, although their formation by *St. thermophilus* β-gal was reported (Toba et al.; 1981). Also, our results were in contrast to the report of Greenberg and Mahoney (1983), who detected only disaccharides and no higher oligosaccharides in the *St. thermophilus* β-gal lactose hydrolyzed skim milk, possibly due to different detection methodologies used. The HPLC patterns of the transferase product formation by all three

examined CCE preparations were similar and the quantitative differences observed were evidently caused by the different rates of the product formation (Figure 6-2 and 6-3).

The formation of oligosaccharides was significantly ($p<0.001$) affected by all examined variables (enzyme origin, lactose concentration, temperature, time). The highest oligosaccharide production was achieved at 50°C in 30% (w/w) lactose solution for all CCEs examined. The St143 CCE produced significantly more ($p<0.05$) total oligosaccharides - including newly formed disaccharides - than the other two CCEs originating from *Lactobacillus* sp. at all lactose concentrations and temperatures studied, reaching maximum oligosaccharide concentration of 60.1 mg mL^{-1} (approx 20% of the total carbohydrates) in 30% (w/w) lactose preparation at 50°C after 120 min (Table 6-4). Toba et al. (1985) and later German et al. (1996) also reported high transferase activity of the *St. thermophilus* β-gal.

The effect of the lactose concentration on the amount of the oligosaccharide formed has been recognized previously (Huber et al., 1976; Iwasaki et al., 1996; Reuter et al., 1999) with the temperature effect appearing to be species dependent (Reuter et al., 1999). In the present study, all three CCE preparations studied followed a similar pattern, achieving the oligosaccharide production maxima at the same temperature (Table 6-4 and 6-5). The maximum oligosaccharide production (approx 20% and 16.5% of the total sugars in lactose and skim milk systems, respectively) achieved by St143 CCE in our study was lower than the values reported previously for *St. thermophilus* β-gal – up to 25% in milk (Greenberg and Mahoney, 1983) or 40% in a buffered lactose solution (Smart, 1991) - but comparable to those for *E. coli* and *K. lactis* β-gal in lactose solutions and skim milk (Mozaffar et al., 1985). Noticeably, the oligosaccharide production was

lower in skim milk than in lactose preparations even at the same (50 mg mL^{-1}) lactose concentration (Figure 6-2 and 6-3; Table 6-4 and 6-5).

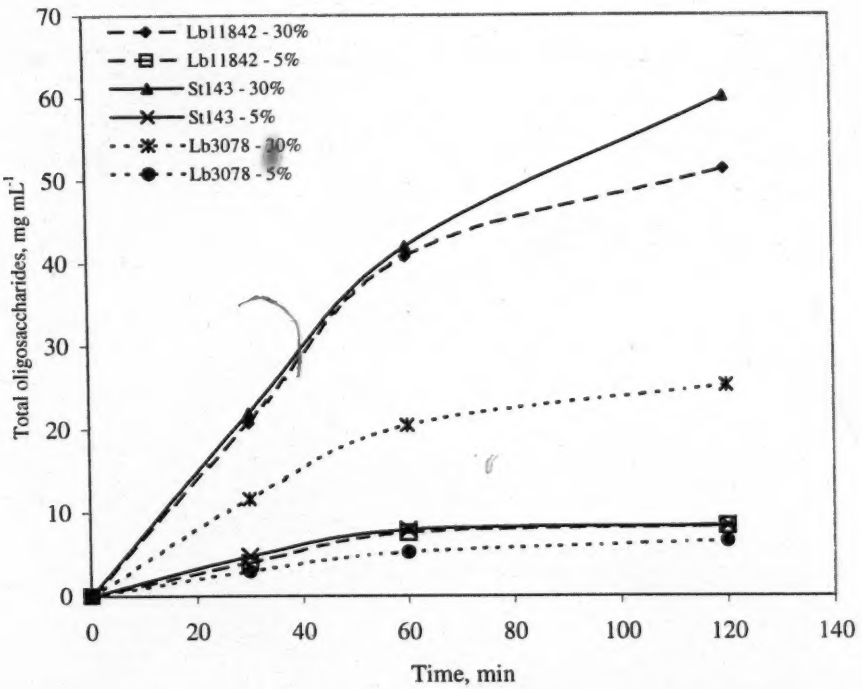

Figure 6-2: Oligosaccharide formation by the β-galactosidase-containing CCEs from three thermophilic dairy cultures during the lactose hydrolysis in 5 or 30% (w/w) buffered (pH 6.8) lactose solution at 50°C.

(Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus 11842*; St143 – *St. thermophilus* 143; Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; error bars showing adjusted standard error of the means are the size of the data marker points)

Although lower in the total amount, the maximum of the oligosaccharides production by the β-gal-containing CCEs was reached sooner (after 60 min) at low (50 mg mL^{-1}) rather than high (300 mg mL^{-1}) lactose concentrations and remained fairly constant till termination (Figure 6-2). Indicatively, the maximum of the oligosaccharide production was not reached for Lb11842 and St143 CCE likely due to insufficient lactose hydrolysis. Prenosil et al. (1987b) correlated the oligosaccharide production to a high

Table 6-4. The content of oligosaccharides formed during lactose hydrolysis in buffered lactose solutions as identified by HPLC analysis after termination of the lactose hydrolysis reactions conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures

Lactose[+]/ CCE[*]	Sugars, mg mL^{-1}											
	Total disaccharides[**]				Trisaccharides				Tetrasaccharides			
	30[***]	40	50	60	30	40	50	60	30	40	50	60
300[+]												
Lb11842	13.07	19.42	22.98	14.67	8.14	16.29	26.83	16.94	1.22	1.31	1.52	1.7
St143	13.37	20.05	25.36	20.52	9.21	18.46	32.77	25.45	1.32	1.36	1.95	1.79
Lb3078	9.61	13.15	15.91	13.55	6.04	9.61	20.42	12.34	0.73	0.73	0.91	1.16
200												
Lb11842	8.17	9.66	14.66	13.47	6.92	11.11	16.75	8.7	1.03	1.02	0.91	1.14
St143	9.59	12.70	15.96	14.72	7.59	11.96	15.83	12.44	1.32	1.06	0.96	1.16
Lb3078	7.38	8.59	12.26	12.90	2.65	7.88	7.59	6.66	0.67	0.67	0.79	0.92
125												
Lb11842	6.35	6.91	8.82	8.71	6.91	6.17	10.03	9.74	1.44	1.04	1.15	0.87
St143	7.09	7.16	10.08	9.75	3.79	7.19	9.13	6.28	1.52	1.04	1.26	0.87
Lb3078	4.95	5.03	7.93	7.73	2.3	5.75	5.96	3.48	0.57	0.57	0.61	0.77
50												
Lb11842	3.13	3.29	3.59	3.52	1.7	2.27	3.76	3.41	0.47	1.11	1.02	0.9
St143	3.33	4.21	4.79	4.08	1.47	2.46	3.05	2.7	0.6	1.19	1.27	0.86
Lb3078	2.97	2.72	3.98	3.21	1.59	1.75	2.33	2.29	0.37	0.37	0.54	0.68
SEM[++]	0.16				0.11				0.13			

[*]CCE – β-galactosidase-containing crude cellular extracts from Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143 – *St. thermophilus* 143, and Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; [**]other than lactose; [***]temperature, °C; [+]lactose concentration, mg mL^{-1}; [++]SEM – adjusted standard error of the mean, n = 4 or more.

Table 6-5. The content of oligosaccharides formed during lactose hydrolysis in skim milk preparations as identified by HPLC analysis after termination of the lactose hydrolysis reactions conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures

Lactose[+]/ CCE[*]	Sugars, mg mL^{-1}											
	Total disaccharides[**]				Trisaccharides				Tetrasaccharides			
	30[***]	40	50	60	30	40	50	60	30	40	50	60
150[+]												
Lb11842	6.67	8.80	10.34	10.09	7.26	9.08	8.4	6.6		ND[++]		
St143	7.15	9.10	12.28	10.40	8	8.98	12.58	9.89		ND		
Lb3078	6.59	8.52	6.25	6.45	3.86	6.6	7.58	8	0.61	0.63	0.73	0.82
100												
Lb11842	3.92	5.59	7.11	5.90	4.51	5.27	5.07	4.82		ND		
St143	4.21	6.73	7.27	6.90	3.74	4.15	5.06	4.81		ND		
Lb3078	3.77	5.37	5.47	2.90	4.29	4.29	4.69	4.81	0.52	0.53	0.68	0.74
50												
Lb11842	2.17	3.61	3.88	3.06	1.14	1.09	1.15	1.38		ND		
St143	3.39	4.01	3.99	3.24	2.2	2.46	2.49	2.38		ND		
Lb3078	2.17	3.41	3.28	2.47	1.93	2.11	2.2	2.38	0.34	0.35	0.5	0.65
SEM[+++]	0.34				0.21				0.05			

[*]CCE – β-galactosidase-containing crude cellular extracts from Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143 – *St. thermophilus* 143, and Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; [**]other than lactose; [***]temperature, °C; [+]lactose concentration, mg mL^{-1}; [++]ND – not detected; [+++]SEM – adjusted standard error of the mean, n = 4 or more.

degree of lactose hydrolysis, achieving maxima when up to 80% of lactose was hydrolyzed. Based on their findings, higher amounts of oligosaccharides might have been produced in our study by prolonging the lactose hydrolysis reaction time.

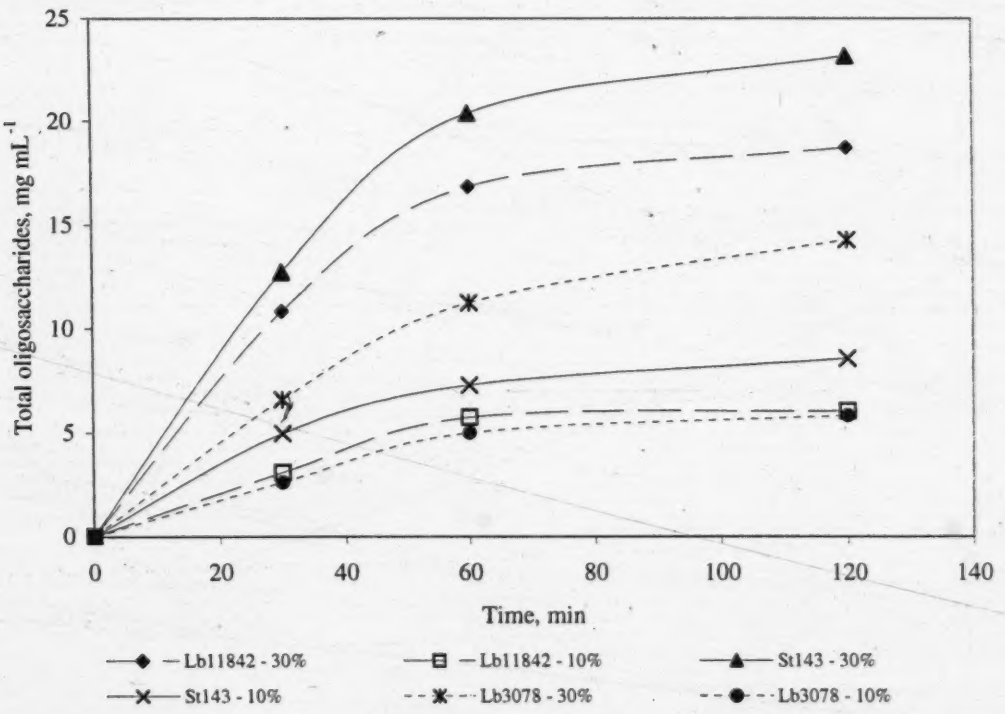

Figure 6-3: Oligosaccharide formation by the β-galactosidase-containing CCEs from three thermophilic dairy cultures during the lactose hydrolysis in 10 or 30% (w/w) skim milk total solids preparation at 50°C.
(Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus 11842*; St143 – *St. thermophilus* 143; Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; error bars showing adjusted standard error of the means are the size of the data marker points)

6.3.3. Proteolytic activity of CCE preparations

All three β-gal-containing CCE preparations exhibited appreciable proteolytic activity in skim milk systems. The proteolytic activity of the CCE preparations resulted in a steady increase of the total surface area under the HPLC peptide peaks over the time of

the hydrolysis reaching maxima at the 120 min reaction time endpoint. Figure 6-4 shows maxima obtained for the proteolytic activities of the three CCEs studied in 10 or 30% skim milk reaction systems after the termination of the hydrolysis. Generally, the values obtained for the 20% skim milk preparations were between the respective data pairs presented in Figure 6-4 (Figure A-7). Although covariate adjustment using the cell paste total solids was significant ($p=0.0329$), the adjusted proteolytic activity still differed significantly ($p<0.05$) among the CCE sources with Lb11842 CCE being the most proteolytic. Such a high capability of the CCE preparations to hydrolyze milk proteins is obviously related to the absence of the purification step during the CCE production (Vasiljevic and Jelen, 2001; 2002a). On the other hand, the substantial activity differences follow from the origin of the CCE preparations. Shihata and Shah (2000) comparing proteolytic activity of several LAB species showed that *Lb. bulgaricus* and *St. thermophilus* strains were highly proteolytic in comparison to *Lb. acidophilus* and *Bifidobacterium* sp. strains.

The concentration of the skim milk total solids had a significant ($p<0.05$) effect on the amount of peptides formed through the proteolysis, higher concentrations resulting in lower proteolytic activity (Figure 6-4). Such effect of the increased skim milk total solids might have resulted from the concomitant decrease in water activity, which could have had an inhibitory effect on the proteolytic activity of the studied CCE preparations (Gobbetti et al., 1999). The proteolytic activity of all CCE preparations clearly showed the temperature dependence, reaching maxima for all three CCE preparations at 40°C, in concert with previously reported findings (Vasiljevic and Jelen, 2002a). Similarly, Abraham et al. (1993) and Fira et al. (2001) reported enhanced proteolytic activity of *Lb.*

bulgaricus around 40°C as opposed to *Lb. acidophilus* strains with maximum at 50°C (Abraham et al., 1993). The differences in temperature optima for all three CCEs used in our study between the proteolysis and transferase reactions give a possible tool for manipulating these activities depending on the desired end-use of the CCE.

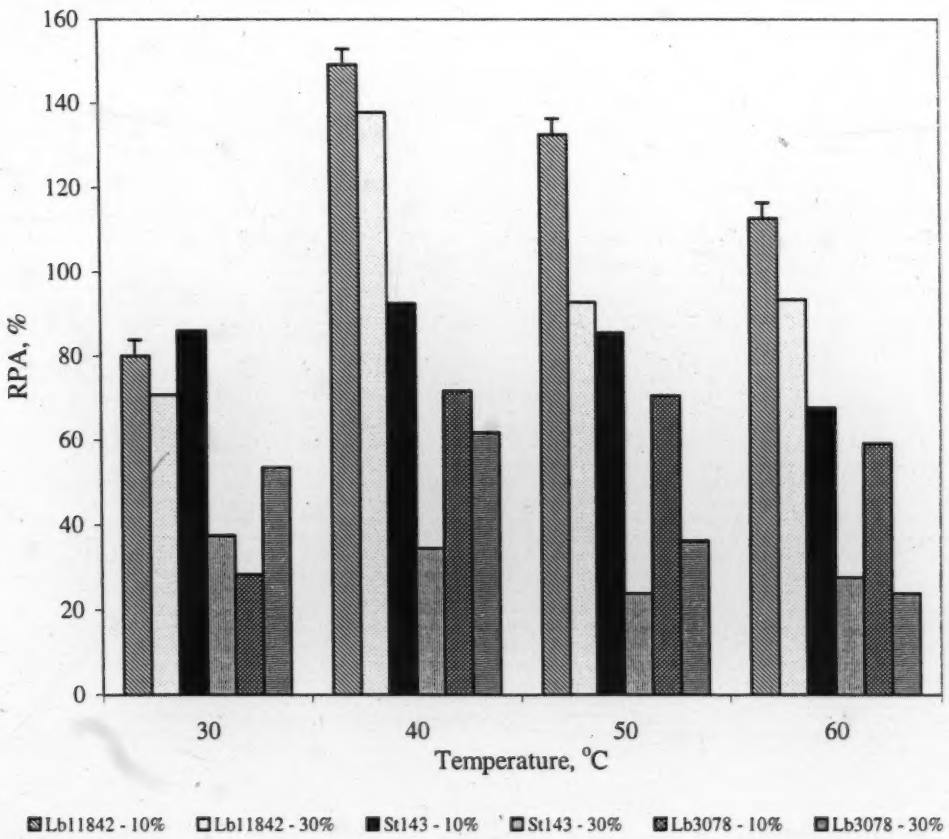

Figure 6-4: Relative proteolytic activity (RPA, %) of the β-gal-containing CCEs from three thermophilic dairy cultures during the lactose hydrolysis in 10 or 30% (w/w) skim milk preparation performed at different temperatures.
(Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus 11842*; St143 – *St. thermophilus* 143; Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; bars present adjusted standard error of the means; n=4 or more)

6.3.4. Optimization of oligosaccharide production and proteolytic activity

The effects of the three independent variables (lactose or skim milk total solids concentration, temperature and reaction time) on the oligosaccharide formation or proteolytic activity of the three studied CCEs were optimized using the response surface methodology approach. Table 6-6 provides the estimates for the second order model coefficients, used to fit the multivariate dependence. Generally, all independent variables and corresponding interactions affected the response of the dependent variables significantly ($p<0.05$) in the lactose-containing systems.

The coefficients of determination indicate that at least 87.5% variability could be explained by the models, thus producing clearly predictive values (Khuri and Cornell, 1996). The ridge analysis, a part of the response surface methodology, was employed for the optimized maximum response determination of the independent variables with the results presented in Table 6-7. The predicted oligosaccharide production of the three studied CCEs had the optima close to experimentally determined value of 50°C. In contrast, the optimized proteolytic activities had maxima around 43°C, slightly above the experimental value. Also, the predicted response of both studied dependent values was lower than experimentally determined.

Figure 6-5 shows the surface response model for the predicted oligosaccharide formation in buffered lactose solutions using *Lb. delbrueckii* ssp. *bulgaricus* β-gal-containing CCE at 50°C. Clearly, the maximum response using the optimization approach was predicted at higher lactose concentration at the end of the reaction period.

Table 6-6. The second-order model coefficients for the prediction of the oligosaccharide formation or relative proteolytic activity during the lactose hydrolysis by CCEs in lactose or skim milk systems as affected by lactose or skim milk total solids concentration (X_1), temperature (X_2) and time (X_3) and corresponding interactions.

Substrate/ CCE*	Coefficients										R^{2**}
	I	X_1	X_2	X_3	X_1*X_1	X_1*X_2	X_2*X_2	X_1*X_3	X_2*X_3	X_3*X_3	
					Oligosaccharides						
Lactose											
Lb11842	-37.76	-0.060	1.297	0.428	NS***	1.8E-03	-1.4E-02	9.1E-04	NS	-2.9E-03	0.875
St143	-24.90	-0.114	1.175	0.192	6.9E-05	2.1E-03	-1.4E-02	1.2E-03	1.4E-03	-2.0E-03	0.931
Lb3078	-11.26	-0.040	0.409	0.160	4.9E-05	7.0E-04	-4.4E-03	5.3E-04	6.3E-04	-1.3E-03	0.946
Skim milk											
Lb11842	-19.749	-0.023	0.792	0.167	8.7E-05	NS	-8.0E-03	1.2E-04	4.6E-04	-8.6E-04	0.954
St143	-13.999	-0.040	0.587	0.198	8.83E-05	3.64E-04	-6.16E-03	1.53E-04	NS	-9.09E-04	0.941
Lb3078	-18.031	-0.001	0.674	0.193	5.9E-05	-2.2E-04	-6.3E-03	NS	-7.6E-04	-6.7E-04	0.897
					Relative proteolytic activity						
Skim milk											
Lb11842	-27.60	0.090	13.348	NS	-2.4E-05	-4.0E-03	-1.3E-01	NS	NS	-5.6E-03	0.897
St143	-61.73	-0.052	3.251	1.130	NS	NS	-3.2E-02	-1.8E-03	-5.1E-03	NS	0.885
Lb3078	-81.65	0.259	7.515	0.643	NS	-7.4E-03	-6.5E-02	NS	NS	-1.3E-03	0.904

*CCE – β-galactosidase-containing crude cellular extracts from Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143 – *St. thermophilus* 143, and Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; **coefficient of determination; ***not significant, α=0.05.

Table 6-7: Predicted maximum of the optimized response for the oligosaccharide formation or relative proteolytic activity during the lactose hydrolysis by CCEs in lactose or skim milk systems

Medium/ CCE*	Predicted response**	Standard error	Concentration mg mL^{-1}	Temperature °C	Time min
			Oligosaccharides		
Lactose					
Lb11842	36.7	0.73	274.6	50.0	90.2
St143	38.8	0.58	270.8	49.4	94.2
Lb3078	20.6	0.26	273.1	49.6	92.4
Skim milk					
Lb11842	14.8	0.18	275.0	48.5	97.2
St143	15.6	0.22	270.8	48.8	99.6
Lb3078	11.2	0.21	274.8	43.9	99.6
			Relative proteolytic activity		
Skim milk					
Lb11842	133.0	2.37	122.5	42.6	108.0
St143	68.2	1.48	133.9	43.7	104.7
Lb3078	60.0	1.56	132.1	41.7	112.2

***CCE – β-galactosidase-containing crude cellular extracts from Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143 – *St. thermophilus* 143, and Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; **predicted oligosaccharide concentration, mg mL^{-1}, or relative proteolytic activity,%.**

The optimized maximum obtained at lower lactose concentration was achieved sooner with subsequent decline towards the end of the reaction time. The response surface model for the predicted proteolytic activity of *Lb. delbrueckii* ssp. *bulgaricus* CCE was plotted as a function of temperature and skim milk total solids concentration at 120 min as the proteolytic activity attained maximum values at the end of the studied period (Figure 6-6). Noticeably, the lower skim milk total solids concentration and intermediate temperatures favor highest proteolytic activity of the CCE. The visualized

response surface models for the optimized dependent variable responses of the other two CCEs are included in the Appendix (Figures A – 8-14).

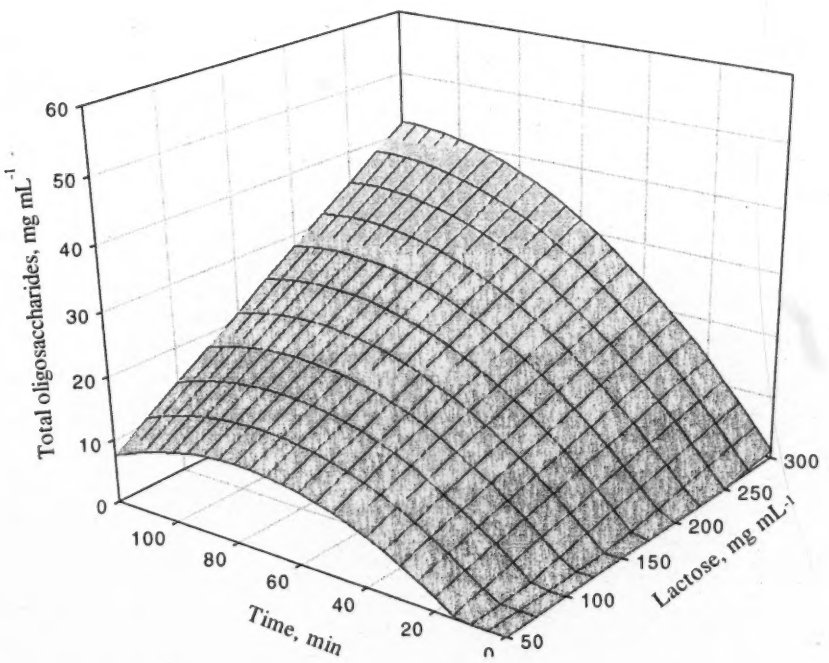

Figure 6-5: Predicted oligosaccharide formation during the lactose hydrolysis in buffered lactose solutions by *Lactobacillus delbrueckii* ssp. *bulgaricus* CCE at 50°C

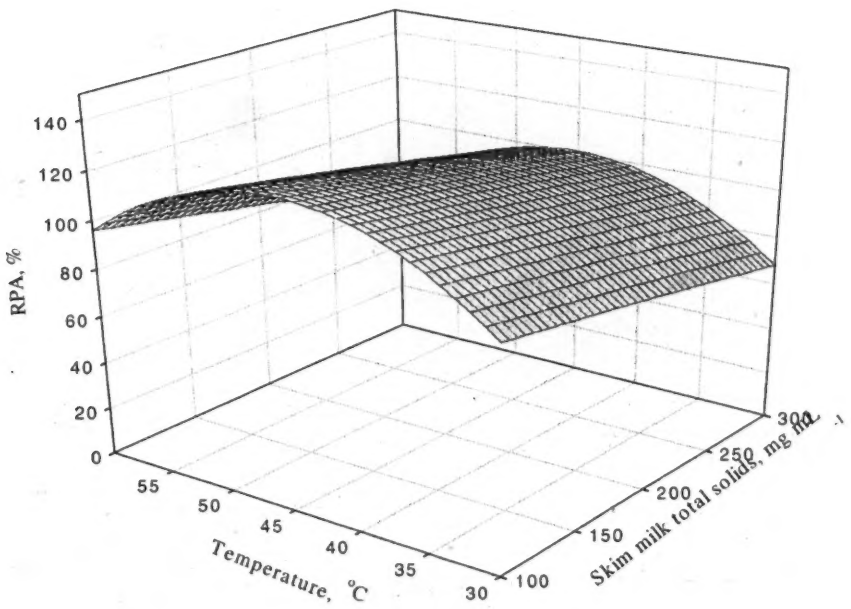

Figure 6-6: Predicted relative proteolytic activity (RPA, %) during the lactose hydrolysis by *Lactobacillus delbrueckii* ssp. *bulgaricus* CCE in skim milk systems at the end of 120 min reaction period

6.4. Conclusions

All β-gal-containing CCE preparations obtained from three thermophilic dairy cultures studied were able to catalyze the formation of oligosaccharides. The formation rate and the amount of oligosaccharides were origin specific while the effects of lactose concentration and temperature were substantial in all cases. The highest oligosaccharide production was achieved by St143 at 30% lactose concentration, followed by Lb11842 and Lb3078 CCEs. The maximum oligosaccharide production (approximately 20% of the total sugars) was reached at 50°C and 30% (w/w) lactose concentration by St143 CCE. The amount of the oligosaccharides formed by all CCE preparations was substantially

lower in skim milk in comparison to corresponding lactose solutions; no tetrasaccharides were detected in skim milk preparations when using Lb11842 or St3078 CCE. The lactose hydrolysis by all CCE preparations resulted in the formation of at least two different disaccharides, likely allolactose and 6-*O*-galactosyl-β-D-galactopyranose. The rate of production of the constitutive monosaccharides achieved by Lb11842 CCE was faster than for the other two enzyme preparations, subsequently resulting in higher k_{cat} values at corresponding temperatures.

Similarly, Lb11842 CCE exhibited higher proteolytic activity than the other two studied CCEs. The maximum proteolytic activity was generally reached at 40°C in 10% skim milk and suppressed at higher concentrations of skim milk total solids (20 or 30% w/w). The characterization and simultaneous optimization of the lactose hydrolysis, oligosaccharide formation and proteolytic activity of different CCEs in various lactose-containing preparations may be important for some industrial applications such as using the CCE preparations as a fermentation enhancer.

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Chapter 7*

Retention of β-galactosidase Activity in Crude Cellular Extracts from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 upon Drying

7.1. Introduction

The interest of the dairy industry in lactose hydrolysis has been driven mainly by the fact that more than 70% of the world's population suffers from the inability to utilize lactose or lactose containing products due to the lactose intolerance symptoms caused by the lack of β-galactosidase activity (Lee and Krasinsky, 1998). The enzyme β-galactosidase (E.C. 3.2.1.23) present in the human gastrointestinal system or used in industrial processing cleaves lactose into constitutive monosaccharides, glucose and galactose. The commercially exploited sources of β-galactosidase (β-gal) have been of microbial origin, mainly yeasts and molds (Holsinger and Kligerman, 1991). Recently, increased attention has been paid to other β-gal GRAS sources, especially thermophilic lactic acid bacteria (LAB) such as *Streptococcus thermophilus* (Greenberg et al., 1985; Chang and Mahoney, 1989) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Kreft and Jelen, 2000; Kreft et al., 2001). The stability, activity and use of β-gal from these strains for lactose hydrolysis have been assessed in buffered lactose solutions, skim milk and whey. Vasiljevic and Jelen (Chapter 3,4, this thesis) explored the possibilities of using crude cellular extracts (CCEs), containing β-gal obtained by mechanical disruption of cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 with little or no

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purification, for lactose hydrolysis in dairy products. However, the investigations of this process approach have been limited so far to the immediate use of CCEs in the aqueous form.

Bioactive materials such as enzymes, bioactive proteins and peptides are unstable in aqueous solutions in the absence of stabilizers (Chang and Mahoney, 1989; Mahoney and Wilder, 1989) and are often preserved by drying. Freeze-drying has been the method of choice for long-term preservation of bioactive materials. However, there are drawbacks associated with this method, such as high cost and limited capacity, in comparison to more industrially feasible methods such as spray drying. The effects of drying methods on the properties of purified or crude β-gal from LAB have not been explored, although this enzyme from other sources served as a model in several studies exploring the drying kinetics during spray or freeze drying (Yamamoto and Sano, 1992; Yoshioka et al., 1993; Yoshioka et al., 1994). Broadhead et al. (1994), investigating the effects of spray drying conditions on the activity retention of β-gal from *Aspergillus oryzae*, reported satisfactory results when drying adjuncts such as trehalose were added.

The effects of dairy ingredients on the retention of enzymatic activity during drying have been studied occasionally. Daemen and Van der Siege (1982) showed that the deactivation of phosphatase, rennin and α-amylase during spray drying of skim milk and whey was mainly a function of the outlet temperature, particle size and initial total solids content. Lactose was efficient in protecting lactate dehydrogenase from thermal stress, while alcohol- and malate-dehydrogenase were deactivated quickly even when lactose was present (Suzuki et al., 1997). Yamamoto and Sano (1992) demonstrated that the activity preservation of β-gal activity from *Aspergillus oryzae* was almost complete in

the presence of lactose. The addition of dried β-gal preparations into the milk powders could alleviate the problem of milk consumption and possibly supply in the area with inhabitants affected with lactose intolerance and low domestic milk production (Palumbo et al., 1995). However, potential deactivation of the β-gal in CCEs and similar crude enzyme preparations resulting from drying has not been investigated.

The overall aim of our study was to determine the effect of spray- and freeze-drying of CCEs from *Lb. bulgaricus* 11842 on the retention of β-gal activity, with the emphasis on the protective effects of drying adjuncts of dairy origin.

7.2. Materials and Methods

7.2.1 Culture cultivation and cell collection

Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842 was obtained from University of Alberta, Department of Agricultural, Food and Nutritional Science. The production of the crude β-gal extracts was described previously (Vasiljevic and Jelen, 2002). Briefly, the stock culture was produced in a 10 h fermentation period at 43±1°C and pH 5.6 maintained by KOH in a 2 L fermentor (New Brunswick Scientific Co., New Brunswick, NJ). The culture was transferred into 50 L of pasteurized commercial skim milk (Lucerne milk processing unit, Edmonton, AB) placed into a 75 L steam jacketed fermentor (LH 2000 series II, Amexon process, Ltd, London, UK) equipped with a temperature controller and a pump (Masterflax, 7534-10, Cole-Palmer Instrument Co., Chicago, Ill) for automatic addition of a neutralizer for pH control. Before the culture addition, the commercial skim milk was heat treated at 80°C for 10 h, to eliminate any microbial contamination while minimizing the detrimental effect on the milk

characteristics. During the fermentation, the pH was maintained at 5.6±0.2 by adding 10 M KOH. The fermentation was terminated after 10 h and the medium was quickly cooled to 10°C by circulating cold water through the fermentor jacket to limit further culture growth and preserve the β-gal activity of the culture. After cooling, the medium was transferred into a 50 L carboy and stored in a cooler at 7°C. The following day, the cells were collected by batch centrifugation at 14000 *x g* at 4°C for 10 min (Beckman model J2-21, Beckman Coulter Inc, Fullerton, CA). The paste obtained was gathered in 500 mL plastic containers and stored at approximately -25°C until required.

7.2.2 Preparation of feedstock solutions and CCE for drying

The basic feedstock solution, used as a control, was a skim milk salt buffer solution (SMSB) (pH 6.8, Santos et al., 1998), with the approximate total milk salts concentration of 0.7% (w/v). The SMSB was used to prepare solutions of 0.8 % (w/v) whey protein isolate (PowerPro whey proteins, lot #27361, Land O'Lakes Inc., St. Paul, MN), as well as 2.7% (w/v) Na-caseinate (Sigma-Aldrich Canada Ltd., CA). Solutions of 6.5% (w/v) whey powder (Dairyworld Foods, Vancouver, BC) in deionized water and commercial pasteurized skim milk (Lucerne) were also used. All feedstock solutions were prepared or acquired when needed and used immediately.

The frozen cell paste (approx. 14% dry matter) was thawed at 37°C in a water bath and recombined with each feedstock solution in the ratio 1: 4 (w/w). The liberation of intracellular β-gal was achieved by passing mixed preparations of cells and the feedstock solutions through a microfluidizer (Model M-110EH, Microfluidics, Newton, MA) equipped with two ceramic disrupting chambers, H30Z and H10Z, with the channel

size of 200 and 100 μ, respectively. The working pressure was set at 172 MPa. The chambers were placed into an ice-water bath to prevent the loss of β-gal activity due to heat generation. The crude cellular extracts (CCEs) thus obtained were kept in a cooler at 7°C and used within 4 hours. A portion of approximately 200 mL was immediately frozen for freeze-drying by placing it in a 250 mL plastic container at approx -25°C in a freezer. The remaining part was divided into three equal portions and processed by spray drying at three different drying temperatures.

7.2.3. Drying of CCE preparations

All spray drying was carried out using a Büchi 190 co-current Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland). Approximately 250 g of a CCE was placed into an ice-water bath equipped with a stirring device to minimize the enzyme activity and/or degradation. Silicone tubing (inner diameter 4 mm) and a peristaltic pump were used to feed the solutions to the 0.5 mm nozzle of the drier. The solution feed rate was adjusted to maintain outlet temperatures of 45, 55 or 65°C with preset inlet temperatures at 130, 140 or 150°C. The air-flow consumption was set at 800±50 L h^{-1} and the aspirator setting maintained at approximately 22.2 kg air h^{-1}, reducing the working pressure in an air cyclone section of the drier by 3.5 kPa. After separation in the air cyclone, dried powders were retained in a glass collector and transferred into 150 mL screw-top plastic containers after completion of each run.

The freeze-drying was conducted using the previously frozen portion of each CCE. Samples were dried in a freeze drier (Virtis model 50-SRC-5 Bulk Sublimator,

Virtis Co., Inc., Gardiner, NY), under vacuum (absolute pressure 66 Pa), with the shelf temperature at 25°C. The drying was terminated after 72 hrs.

A schematical representation of the preparation of feedstock mixtures and their processing is depicted in Figure 7-1.

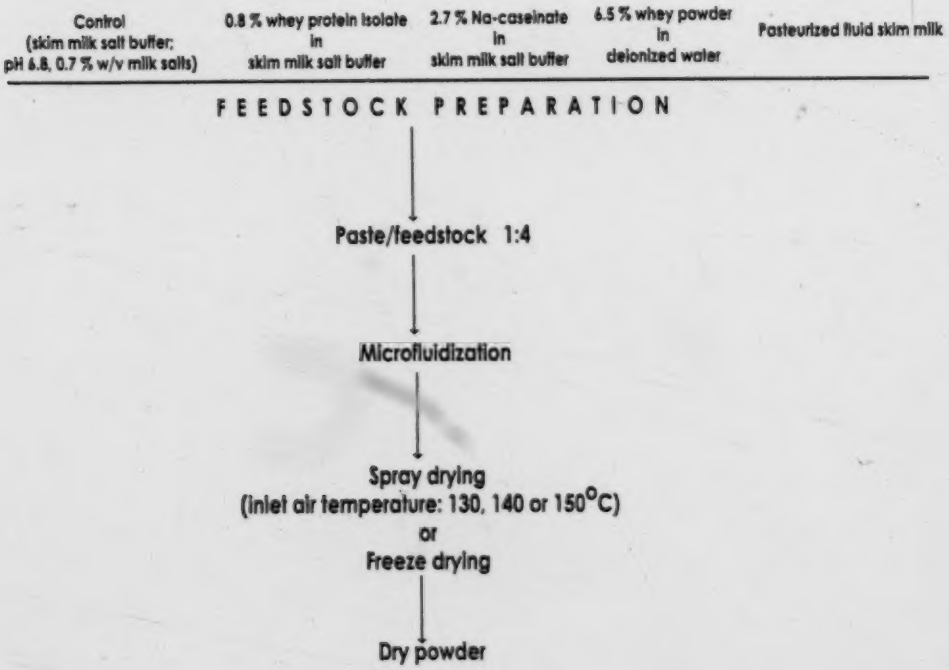

Figure 7-1: Schematic presentation of the preparation and processing of feedstock mixtures

7.2.4. Determination of β-galactosidase activity and moisture content

The retention of β-gal activity upon drying was evaluated from the activity determined in CCE solutions prior to drying and reconstituted dry powders immediately after drying. The retention was calculated as:

$$\text{Retention},\% = \frac{N}{N_0} \cdot 100$$

Formula 7.1

where: N - β-gal activity in dry powders and N_0 - β-gal activity in the processed mixture before drying, both expressed as μmol of *o*-nitrophenol (ONP) released, per g dry matter.

For the determination of β-gal activity in the processed mixtures prior to drying, fresh samples were diluted 10 fold and the activity was assessed by the ONPG method as previously reported (Chapter 3, this thesis). The dry CCE powders were redissolved in 10 mL of deionized water to form solutions of concentrations close to diluted fresh samples. After ONPG hydrolysis and prior to the spectrophotometric determination, all samples were centrifuged at 1500 *x g* for 10 min for removal of insoluble solids that would interfere with the spectrophotometric reading. The β-gal activity was expressed per g total solids of the CCE preparations to account for the slight difference in concentrations between fresh and reconstituted samples. The moisture content of the fresh CCE preparations and dry powders were determined gravimetrically by air drying at 105°C for 12 hrs.

7.2.5. Statistical analysis

All experimental drying trials were at least replicated and all subsequent analyses were carried out at least in duplicate resulting in n = 4 or more. The data were analyzed as a full factorial design, using the General Linear Model of SAS, including main effects and all corresponding interactions (SAS Institute, 1992), and a Tukey's test was used to

separate means. The covariate analysis using the initial total solids content as a covariate and regression analysis were also employed. Statistical significance was set at $\alpha = 0.05$. All results are expressed as a mean ± standard error of the mean (SEM) or ± standard deviation.

7.3. Results and Discussion

7.3.1. Effect of drying method on the β-galactosidase activity retention

The stabilization of pure enzymatic preparations is usually achieved by freeze-drying. Spray drying, often regarded as a 'harsh' method due to high temperature of the drying gas, has been used for drying of bioactive proteins with varying success (Werner et al., 1993; Broadhead et al., 1994; Mumenthaler et al., 1994). Little is known about the effects of drying methods on the preservation of the β-gal activity from LAB sources, especially in complex systems such as CCE. By preserving the β-gal activity in dry CCE powders, the possibilities of using crude β-gal preparations in further investigation or in industrial applications of the novel technology (Vasiljevic and Jelen, 2001) could be expanded.

As seen in Figure 7-2, both drying methods had a detrimental effect on the enzyme activity retention in the basic CCE preparation, in which only the SMSB was used for diluting the cellular paste for the cell disruption. On an average, 90% of the activity was lost in the control samples. To improve the retention of β-gal activity, the incorporation of the enzyme into some dairy based systems was explored as a possible approach. The presence of whey proteins in fresh CCE preparations did not change the retention of activity in spray dried samples significantly ($p>0.05$), while the addition of

sodium caseinate, although showing a statistically significant ($p<0.05$) improvement of the enzyme preservation in comparison to the control, still resulted in 80% loss of activity. On the other hand, when the CCE was prepared using whey or commercial skim milk, the retention of β-gal activity upon spray drying was much greater ($p<0.001$).

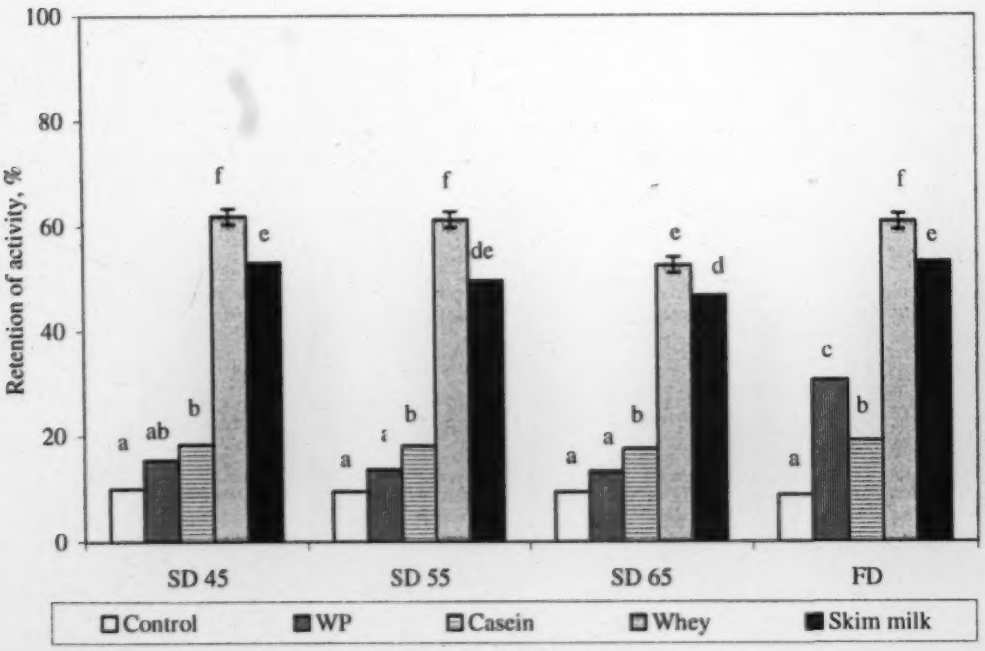

Figure 7-2: The effect of the addition of skim milk components, whey and skim milk on the activity retention of the crude β-galactosidase extract from *Lb. bulgaricus* 11842 upon spray drying at three different outlet temperatures and freeze drying

(Control – CCE in skim milk salt buffer (SMSB); WP - whey protein – 0.8% (w/v) of WPI in SMSB; Casein – Na-caseinate - 2.7% (w/v) in SMSB; Whey – whey powder - 6.5 (w/v) in deionized water; Skim milk – commercial skim milk; SD – 45, 55 and 65 – spray drying and corresponding outlet temperatures; FD – freeze drying. Error bars show the adjusted standard error of the mean (±1.483); $R^2 = 0.984$. Mean bars marked with different letters are significantly different, $\alpha = 0.05$).

Surprisingly, the freeze-drying of the CCE preparations did not improve significantly ($p<0.05$) the preservation of the enzyme and practically identical results were obtained in comparison to spray drying at 45 and 55°C outlet air temperatures. The

only significant ($p<0.05$) improvement in the enzyme protection in freeze-drying in comparison to spray drying was achieved for CCEs preparation containing whey proteins, but it was still significantly ($p<0.05$) lower than for preparations with whey or skim milk, either spray or freeze dried. Daemen and Van der Siege (1982) claimed that the enzyme deactivation during spray drying of skim milk was mainly a function of the outlet temperature, particle size and initial total solids content; lower initial total solids content predominantly resulted in lower enzyme activity retention. The initial total solids concentration of CCE preparations in our study was lower than in the above mentioned study and ranged between 3.9 and 10.2% (±0.21%) (w/w). Within this range, the initial total solids content showed no effect ($p=0.4250$) on the preservation of β-gal activity as revealed by the covariate statistical analysis.

Broadhead et al. (1994), studying the effects of process and formulation variables on the retention of β-gal (*Aspergillus oryzae*) activity during spray drying, reported between 30 and 65% activity loss in the absence of stabilizers, using similar processing variables as in our study. Branchu et al. (1999) reported 75% activity retention of the β-gal originating from *Aspergillus oryzae* in the control preparation (4% w/v total solids) upon spray drying at 60°C outlet air temperature. Substantially lower β-gal activity retention obtained in our study than that achieved in these two studies might have been related to the presence of mineral salts in the basic CCE preparation.

The higher β-gal activity after drying achieved by addition of whey and skim milk might have been caused by the lactose present at similar levels (approximately 5% w/v), in these two feedstock solutions. In contrast, the solutions of the Na-caseinate or the WPI powder, which contained practically no lactose, showed a negligible protective effect.

Also, whey proteins exerted substantially higher enzyme activity preservation upon freeze-drying than upon spray drying. While the whey proteins might have provided some protection during the freezing step of the freeze-drying process, the loss of the protective ability might have resulted from the heat denaturation of whey proteins during spray drying.

Greenberg et al. (1985) reported enhanced thermostability of β-gal from *Streptococcus thermophilus* in milk and sweet whey due to contributions by all major milk constituents. The extent of stabilization was chiefly governed by lactose binding to the enzyme. Chang and Mahoney (1989), on the other hand, found that lactose, while stabilizing in frozen state, destabilized the same enzyme stored unfrozen. They also reported stabilizing effect of whey proteins on β-gal in a buffered solution, although the mechanism could not be explained. Arakawa and Timasheff (1982, 1985) and Arakawa et al. (1990) showed that some osmolytes such as sugars, polyols and different salts stabilize different proteins by preferential hydration, preventing heat- and cold-induced denaturation in solution. However, during dehydration, as in case of freeze drying, the enzyme protection by drying adjuncts appeared to be rather limited and only particular components such as certain sugars (Carpenter et al., 1987; Carpenter et al.; 1988; Yamamoto and Sano, 1992) or salts (Werner et al., 1993) exerted protective action. Results in our study illustrated that the loss of the β-gal activity was profound in the absence of drying adjuncts (Figure 7-2). The significantly higher activity retention of β-gal achieved in whey and skim milk was presumably due to the presence of lactose.

7.3.2. Effect of final moisture content on the β-galactosidase activity

The main objective of drying a food product or a biologically active system is removal of moisture to a level that would provide best preservation of quality and functionality during prolonged storage. The moisture content should be decreased to the optimum point corresponding to the water monolayer value (Labuza, 1980). Broadhead et al. (1994) reported that the best activity retention of dried β-gal preparation during prolonged storage was achieved in the range 2 to 5% of initial moisture content in dry powders.

The moisture content of the various CCE preparations before drying and in the dry powders obtained during the somewhat arbitrarily selected drying conditions are presented in Table 7-1. The kind of protective adjuncts and the temperatures used in the spray drying had significant ($p<0.001$) effect on the residual moisture in dry powders. Generally, the residual moisture levels in whey and skim milk CCE powders obtained at all spray drying temperatures were lower in comparison to other CCE preparations. Schuch et al. (1999) reported difficulties in removing moisture from a whey protein or native casein solution in the presence of different mineral salts during spray drying. Higher salt to total solids ratio in the control, whey or sodium caseinate CCE preparations in our study might have resulted in higher water retention and subsequently higher residual moisture in obtained spray dried CCE powders in comparison to whey or skim milk spray dried CCE mixtures. The residual moisture had no statistically significant effect ($p=0.5946$) on the retention of β-gal activity immediately after drying indicating again a substantial effect of the composition of a CCE preparation.

Table 7-1: Moisture content of CCE preparations from *Lb. bulgaricus* 11842 containing β-galactosidase before and after spray- or freeze-drying.

CCE preparation*	Moisture content, %				
	Before drying	After drying			
		SD - 45	SD - 55	SD - 65	FD
Control	96.1±0.23	9.6±0.95	7.5±0.40	6.6±0.21	4.3±0.23
WP	95.6±0.16	5.4±0.61	4.9±0.41	3.9±0.81	4.4±0.43
Casein	94.0±0.23	7.8±0.76	6.5±0.87	5.5±0.15	3.3±0.52
Whey	92.3±0.12	2.7±0.77	2.1±0.67	1.5±0.38	3.8±0.65
Skim milk	89.8±0.02	2.5±0.38	1.9±0.23	1.3±0.29	3.4±0.29

***Control – CCE in skim milk salt buffer (SMSB); WP - whey protein isolate – 0.8% (w/v) in SMSB; Casein – Na-caseinate - 2.7% (w/v) in SMSB; Whey – whey powder - 6.5 (w/v) in deionized water; Skim milk – commercial skim milk; SD – 45, 55 and 65 – spray drying and corresponding outlet temperatures; FD – freeze drying; Result presented as means ± standard deviation; SEM - ±0.205.**

Apparently the lower residual moisture (below 5%) could be achieved by increasing the spray drying temperature. The employment of such drying conditions is however restricted by the need to retain a high enzyme activity in dry powders which in turn could be achieved by selecting appropriate drying adjuncts. Furthermore, the effects of the final moisture content as well as of the various drying adjuncts on the stability of β-gal upon prolonged storage still remain unknown and need to be explored.

7.4. Conclusions

Both spray drying and freeze-drying had a highly detrimental effect on the activity retention of β-galactosidase in the CCE preparation in the absence of drying adjuncts, with approximately 90% losses of the activity. The addition of whey proteins at

the concentration equal to that found in milk did not improve the enzyme preservation in the spray drying, while the presence of Na-caseinate, likewise at the level corresponding to the casein content of milk, resulted in approximately two-fold increase of the enzyme activity retention. In freeze-drying, the whey proteins showed a significantly better protection than the Na-caseinate with approximately 30% activity retention.

The CCE prepared in whey or skim milk retained substantially more (over 60%) β-galactosidase activity in comparison to all other CCE preparations. This effect could not be attributed to the difference in initial total solids content of CCEs prior to drying and appears to have been related to the presence of lactose in whey and skim milk CCE preparations. The lower temperature (45 and 55°C) of the exit air during spray drying resulted in a similar retention of the enzyme activity as obtained by freeze-drying, while the 65°C substantially lowered the retained β-galactosidase activity in whey and skim milk CCEs as compared to freeze dried preparations. The residual moisture content in CCE powders was substantially affected by type of drying adjunct used and outlet air temperature during spray drying. The desired residual moisture level (below 5%) in CCE powders was achieved for all preparations by freeze-drying, but only for whey and skim milk CCE powders at all temperatures investigated during spray drying. Further investigation is required to elucidate the effect of lactose on the activity retention of β-galactosidase in CCE preparations and to clarify the influence of different drying adjuncts as well as processing parameters employed during drying on storage stability of the dried CCE preparations.

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Chapter 8*

Drying and Storage of Crude β-galactosidase Extracts from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842

8.1. Introduction

The lactose hydrolyzing effectiveness of β-galactosidase (β-gal, E.C. 3.2.1.23) preparations obtained as crude cellular extracts (CCEs) from mechanically disrupted thermophilic lactic acid bacteria has been demonstrated (Jelen, 1993; Kreft and Jelen, 2000; Kreft et al., 2001; Vasiljevic and Jelen, 2002a). The applicability of the proposed approach (Vasiljevic and Jelen, 2001) would be further expanded by drying of the CCE if the β-gal activity would not be affected by the processing or storage. Freeze-drying has been a method of choice for the long-term preservation of bioactive materials, such as enzymes, which are unstable in aqueous solutions (Carpenter et al., 1987; Carpenter and Crowe, 1988). In a series of preliminary experiments, Vasiljevic and Jelen (2002b) investigated the effects of drying conditions on the β-gal activity retention in the CCEs during spray and freeze-drying. In both cases, the activity retention was improved after addition of drying adjuncts of dairy origin, presumably due to the lactose present. Carpenter et al. (1987) and Yamamoto and Sano (1992) reported protection of labile enzymes by certain carbohydrates upon stress such as that caused by the heat in the case of spray drying, or by solute concentration and pH increase during freezing and freeze-

* A version of this chapter has been accepted for publication: Vasiljevic T. and P. Jelen (2003). *Innovative Food Science and Emerging Technologies.*

drying. Similarly, Broadhead et al. (1993) observed enhanced β-gal activity retention during spray-drying by trehalose addition.

The enzymes in aqueous solutions are generally stabilized by osmolytes, such as sugars, some amino acids and salts due to preferential exclusion (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1985). However, the mechanism of the enzyme protection from denaturation and subsequent loss of activity during dehydratation appeared to be rather complex. Depending on the kind of stress imposed, two theories have been proposed: the water replacement (Carpenter and Crowe, 1988) and the glassy state (Izutsu et al., 1994). The effect of skim milk or individual skim milk constituents on the activity retention of different enzymes during drying has not been investigated thoroughly. Lactose showed enzyme stabilizing ability in several studies, mainly due to readily attainable amorphous form (Yamamoto and Sano, 1992; Suzuki et al., 1997; Millqvist et al., 1999a,b). Daemen and Van der Siege (1983) reported enhanced preservation of activity of several enzymes upon spray drying due to increased initial skim milk total solids content. Addition of whey proteins in a concentration corresponding to that found in skim milk substantially improved the enzyme preservation upon freeze drying but was ineffective during spray drying (Vasiljevic and Jelen, 2002b).

The overall aim of the present study was to explore the consequences of spray and freeze drying on the β-gal activity from *Lb. bulgaricus* 11842 in dried CCE preparations, with the emphasis on: 1) the effectiveness of drying adjuncts of dairy origin for the enzyme activity preservation; 2) the role that sugars may play in the mechanism of the β-gal activity protection; and 3) the effect of storage on the stability of the β-gal activity.

8.2. Materials and Methods

8.2.1 Culture cultivation and cell collection

Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842 was obtained from University of Alberta, Department of Agricultural, Food and Nutritional Science. The cell paste was produced as previously reported (Chapter 4 and 7, this thesis). The inoculum for the main fermentation run was prepared by culturing sterilized commercial skim milk for 10 hours at 43±1°C and pH 5.6, maintained by KOH, in a 2 L fermentor (New Brunswick Scientific Co., New Brunswick, NJ). The culture was transferred into a 50 L of pasteurized skim milk (Lucerne milk processing unit, Edmonton, AB) placed into a 75 L steam jacketed fermentor (LH 2000 series II, Amexon process, Ltd, London, UK) and the fermentation was performed under the same conditions as described above. Before the fermentation, the skim milk was heat treated at 80°C for 10 hrs. Upon completion, the fermented milk was cooled, transferred into a 50 L carboy and stored in the cooler at 7°C. Following day, the cells were collected by batch centrifugation (Beckman model J2-21, Beckman Coulter Inc, Fullerton, CA) of approx 2.5 L of the medium at a time at 14000 x g and 4°C for 10 min. The obtained cell paste was gathered as 500 g portions, frozen and stored till needed at -35°C.

8.2.2 Preparation of feedstock solutions and crude cellular extracts (CCEs)

The basic feedstock solution used for preparation of control CCE samples was a skim milk salt buffer solution (SMSB), with the approximate total salt concentration of 0.7% (w/v) and pH 6.8 (Santos *et* al., 1998). The SMSB was used to prepare solutions of

lactose (Fisher Scientific Limited, Nepean, ON, CA), whey protein concentrate (WPC, Promil, Novy Bydžov, Czech Republic) or whey protein isolate (WPI, PowerPro whey proteins, lot #27361, Land O'Lakes Inc., St. Paul, MN). The final concentrations of these solutes in the corresponding CCE feedstock preparations were 5, 10 or 15% (w/w). Low temperature skim milk powder (Dairyworld Foods, Vancouver, BC) was reconstituted in deionized water to prepare solutions giving 10, 20 or 30% (w/w) final total skim milk solids content in these CCE preparations. The compositional data on the dry skim milk and whey protein powders as indicated by manufacturers' specifications are presented in Table 8-1. All solutions were prepared when needed and used immediately.

Table 8-1: The average composition of skim milk, WPC and WPI powders* (% w/w, dry basis) according to manufacturers' specifications

Component	Skim milk powder	WPC	WPI
Protein	37.5	67.7	92
Lactose	53.1	23	<1
Fat	<1	3.8	<1
Ash	8.5	3	4

*Skim milk powder - Dairyworld foods, Vancouver, BC; WPC - Promil, Novy Bydžov, Czech Republic; WPI - PowerPro whey proteins, lot #27361, Land O'Lakes Inc., St. Paul, MN.

The frozen cell paste (approx. 14% dry matter) was thawed at 37°C in a water bath. To avoid problems associated with high viscosity of the concentrated solutions during disruption, the paste was mixed with SMSB or 10% skim milk total solids solution. The CCEs containing the liberated intracellular β-gal were obtained by passing these mixtures through a microfluidizer (Model M-110EH, Microfluidics, Newton, MA) using the setup previously reported (Chapter 7, this thesis). The CCEs obtained were recombined with

prepared drying adjunct solutions resulting in the final concentrations in the CCE feedstock preparations as stated above. The concentration of the disrupted cell paste solids was approximately 3.5% (w/w) in all feedstock preparations. The feedstock mixtures were kept in a cooler at 7°C and used within 4 hours. The preparation and processing of feedstock mixtures is schematically presented in Figure 8-1.

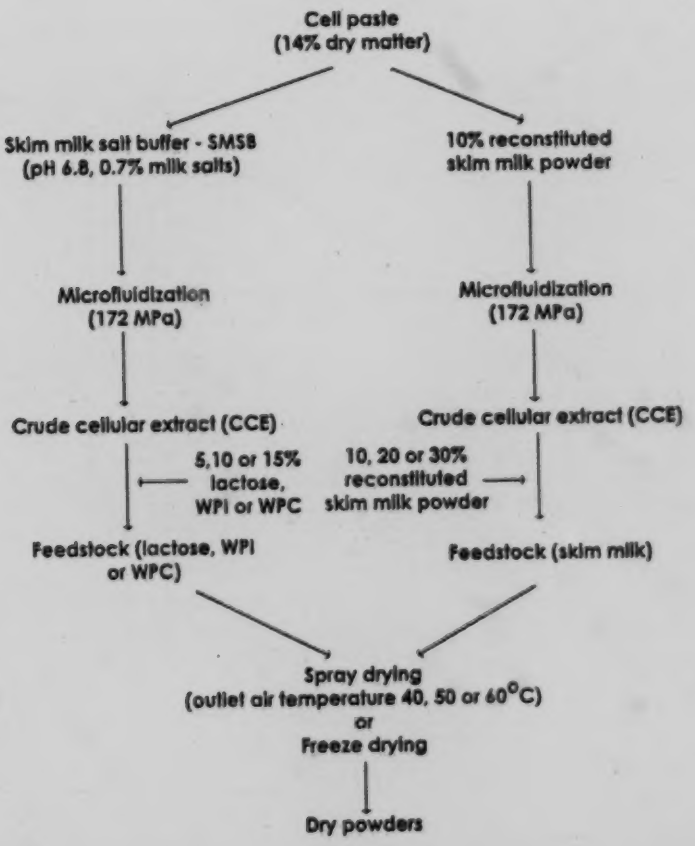

Figure 8-1: The flow diagram of the preparation and processing of feedstock mixtures
(WPI and WPC – whey protein isolate and concentrate, respectively)

8.2.3. Spray drying, freeze drying and storage of dried powders

Spray drying experiments were performed using a Büchi 190 co-current Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland). The feedstock mixtures were processed at a constant feed rate of 0.18±0.06 L h^{-1} with the outlet air temperature monitored and maintained at 40, 50 or 60±1°C, which resulted in the inlet air temperatures of 89±2, 102±2 or 114±2°C, respectively. The air-flow consumption of 800±50 L h^{-1} and the aspirator setting of approx 22.2 kg air h^{-1} were also kept constant. After separation in the cyclone and collection in the system glass collector, dried powders were transferred into 150 mL screw-top plastic containers after completion of each run and stored in a cooler at 7°C.

The freeze-drying of all CCE preparations was carried out in a freeze drier (Virtis model 50-SRC-5 Bulk Sublimator, Virtis Co., Inc., Gardiner, NY). Previously frozen portions were placed on the freeze drier shelf with the temperature maintained at 25°C. Dry powders, obtained under vacuum of absolute pressure 66 Pa after 72 hrs, were collected, packaged and stored in a cooler at 7°C as described above.

8.2.4. The role of sugars in the β-galactosidase protection

In an attempt to confirm the apparent role of lactose in the β-gal protection, cellobiose (4-O-β-D-glucopyranosyl-D-glucopyranose), with a molecular structure similar to lactose, and sucrose (α-D-glucopyranosyl-β-D-fructofuranoside), were compared to lactose in a separate set of drying experiments. The effect of the selected sugars (all from Fisher) at two different concentrations (5 and 10% w/w) on the preservation of β-gal

activity was examined upon spray drying (outlet air temperature at 60±1°C) and freeze-drying using the experimental setup as described above.

Before the drying experiments, the possible interference of cellobiose with the β-gal lactose hydrolyzing activity determination was investigated. The frozen cell paste was thawed, resuspended in 0.01 M potassium buffer pH 6.8 resulting in approximately 10% total solids and the CCE was produced by microfluidization as described above. The possibility that the β–gal could hydrolyze cellobiose was tested by mixing the CCE with a lactose or a cellobiose solution, prepared in 0.01 M potassium phosphate buffer (pH 6.8). The final concentrations of the CCE and the sugars were 2% and 5% (w/v), respectively. The hydrolysis was performed at 50°C for 2 hrs and was terminated by alcohol precipitation (Vasiljevic and Jelen, 2002a). Similarly, the CCE was added to buffered lactose solutions ranging from 2.9 to 294 mM concentrations, with or without cellobiose added at a 2% (w/v) final concentration. In this case, the lactose hydrolysis was stopped by alcohol precipitation after 15 min for the determination of the initial reaction rates. The concentrations of monosaccharides formed during both trials were determined by HPLC, equipped with Shimadzu Ezchrom Chromatography processing system and a Supelcosil LC-NH_2-5 μm column (Supelco, Bellefonte, PA), 25 cm length and 4.6 mm diameter. Elution was accomplished using a gradient of two mobile phase solvents: deionized water and acetonitrile (HPLC grade). The concentration of acetonitrile was decreased linearly over thirty five minutes from 90 to 65% then increased to 90% at 37 min. The total run time was 40 min. Total flow rate was constant at 1 mL min^{-1} and maintained by a Varian 9010 Solvent Delivery System (Sunnyvale, CA). A 500 ELSD (Evaporative Light-Scattering Detector, Alltech, Mandel Scientific

Co., Ltd, Guelph, ON), which evaporated the solvent at 125°C, was used for the detection. A 25 µL sample was injected by a Hewlett Packard Series 1050 autosampler (HP, Mississauga, ON). The peak identification and concentration determination were achieved by using external standard solutions of glucose and galactose and corresponding calibration curves.

8.2.5. Determination of the β-galactosidase activity, moisture content and water activity

The retention of β-gal activity upon drying and storage was evaluated in comparison to the initial enzyme activity determined in the final CCE preparations immediately prior to drying. For the β-gal activity, fresh samples were diluted 10 fold and the activity was assessed by the o-nitrophenyl-ß-D-galactopyranoside (ONPG) method (Vasiljevic and Jelen, 2001). Dry powders immediately after drying and after 15 and 30 day storage at 7°C were reconstituted in 10 mL deionized water to obtain the same concentrations as the diluted fresh samples. Prior to the spectrophotometric determination, all samples, fresh and reconstituted, were centrifuged at 1500 x g for 10 min after the ONPG hydrolysis to remove insoluble solids that would interfere with the reading. The β-galactosidase activity was expressed (in both cases) per g of CCEs total solids to account for the possible slight differences in concentrations between fresh and reconstituted samples. The retention was calculated as:

$$\text{Retention},\% = \frac{N}{N_0} \cdot 100$$

Formula 8.1.

where: N - β-galactosidase activity in dry powders and N_0 - β-galactosidase activity in CCE solutions before drying, both expressed as μmol of *o*-nitrophenol (ONP) released, per g CCE dry matter.

The moisture content of fresh CCE preparations and of the dry powders were determined gravimetrically by air drying at 105°C for 12 hrs. The water activity (a_w) of the powders was determined immediately after drying and at the end of the 30 day storage period. A several gram sample was placed in a plastic cup and loaded into an osmometer (Aqua Lab CX-2, Decagon Devices, Inc., Pullman, WA). The reading was taken after equilibration. All measurements were performed in triplicate.

8.2.6. Statistical analysis

All experimental drying trials were at least replicated with freshly prepared CCE feedstock solutions and all subsequent analyses were carried out at least in duplicate resulting in n = 4 or more. The data were analyzed as a full factorial design, using the General Linear Model of SAS, including main effects and all corresponding interactions (SAS Institute, 1992), and the Tukey test was used to separate means. Regression analysis was also used where necessary. Statistical significance was set at $\alpha = 0.05$. All results are expressed as a mean ± standard error of the mean (SEM).

8.3. Results

8.3.1. The effect of drying adjuncts on the retention of β-galactosidase activity

The preservation of the β-gal activity was greatly enhanced by the presence of lactose in the CCE mixtures (Figure 8-2). The control preparation (CCE in SMSB) lost between 80 and 90% of the initial β-gal activity in the absence of any other drying adjunct. In contrast, over 90% of the original enzyme activity was retained after spray drying at 40 and 50°C outlet air temperature with no significant difference ($p>0.05$) between lactose and skim milk solids at approximately equal lactose concentrations. Although remaining appreciably high, the retention of the enzyme activity was significantly ($p<0.05$) diminished at 60°C at all concentrations of lactose as the adjunct. However, the addition of skim milk solids in all examined concentrations preserved the enzyme activity almost completely at all outlet air temperatures (Figure 8-2; the almost identical data for 20% skim milk total solids concentration shown in Appendix, Figure A-15).

The spray drying of CCE preparations containing whey protein products resulted in significantly ($p<0.05$) lower retention of β-gal activity in comparison to lactose or skim milk CCE preparations. The preservation of the enzyme was substantially improved by increasing the whey protein concentrations to 10 or 15% in these CCE preparations (Figure 8-2; data for 10% lactose and whey protein products concentration was almost identical to the data for 15% lactose, shown in Appendix, Figure A-15). Also, the use of WPC resulted in significantly ($p<0.05$) higher preservation of the enzyme activity at higher temperatures (50 and 60°C) in comparison to WPI.

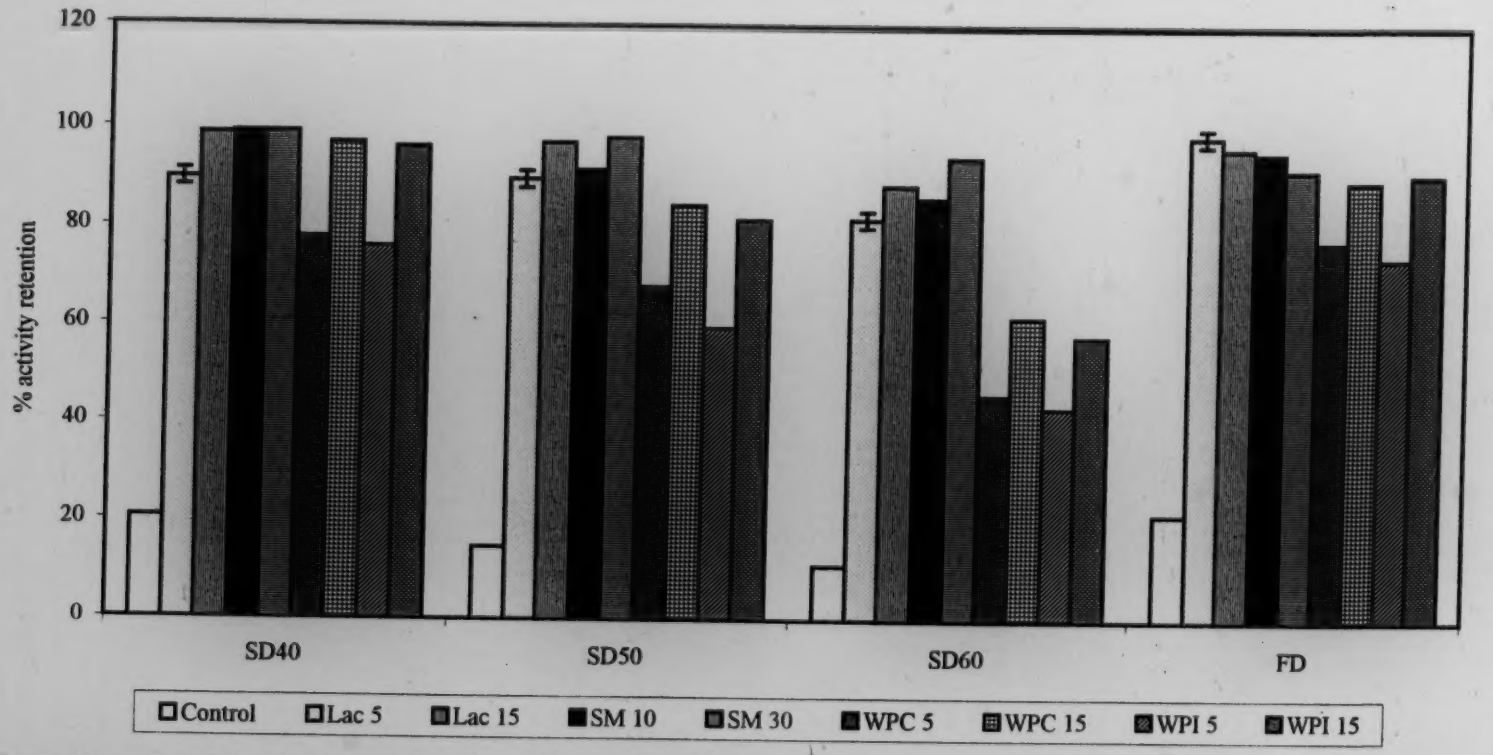

Figure 8-1. The effect of drying methods on the β-galactosidase activity retention in CCE powders from *Lb. bulgaricus* 11842 upon addition of 5% or 15% (w/v) of dairy drying adjuncts. (Control – CEE in skim milk salt buffer (SMSB); Lactose (Lac), WPC and WPI – 5 and 15% final concentration (w/v) in SMSB; SM – 10 and 30% final concentration of skim milk solids (w/v) in deionized water, included based on approx. 5 and 15% lactose content; Bars present the adjusted standard error of the mean, ±1.695).

In both cases, this may be related to the lactose content, which was very low in the WPI preparations (Table 8-1).

Freeze drying of the control SMSB CCE preparations resulted in significantly ($p<0.05$) higher retention of enzyme activity as compared to that processed by spray drying (Figure 8-2). The loss of activity (80%) was still statistically significant ($p<0.05$) and very substantial in comparison to freeze dried CCE preparations containing protective compounds. The β-gal activity in freeze dried CCEs with lactose and skim milk solids were similar to that of the spray dried counterparts obtained at all temperatures. Freeze-dried CCEs containing WPC or WPI resulted in significantly higher ($p<0.05$) preservation of activity than those spray-dried at 50 and 60°C.

8.3.2. The effect of different sugars on the β-galactosidase activity protection

The results presented above appear to confirm our preliminary data (Vasiljevic and Jelen, 2002b) as well as findings by Yamamoto and Sano (1992) highlighting the essential role of lactose for the β-galactosidase activity retention during spray drying. In an attempt to elucidate the mechanism of the β-gal protection by lactose, two different sugars were examined in a new series of experiments: cellobiose, as a lactose analog, and sucrose, widely recognized as a protein stabilizer (Lee and Timasheff, 1981) due to its glass-forming ability (Millqvist-Fureby et al., 1999a). Prior to drying experiments, the suitability of cellobiose in the context of these investigation was examined. The kinetic parameters, K_m and V_{max}, obtained during lactose hydrolysis by CCE preparation containing β-gal in the presence of 2% (w/v) cellobiose (Table 8-2) were not significantly different ($p>0.05$) from those obtained for pure lactose solutions, pointing out the absence

of competitive β-gal inhibition by cellobiose or interference with the determination of β-gal activity. In a separate experiment, cellobiose alone was not hydrolyzed by β-gal during a 2 hr period at 50°C as determined by chromatographic analysis.

Table 8-2: The estimation of kinetic parameters V_{max} and K_m in Michaelis-Menten type kinetics for the lactose hydrolysis by β-galactosidase in the presence or absence of 2% (w/v) cellobiose

Substrate	V_{max}, mM min^{-1}	K_m, M
Lactose	1.37	0.084
Lactose + cellobiose	1.27	0.052
SEM*	0.18	0.012

(* adjusted standard error of the mean, n = 4)

The spray drying experiments with all sugar-containing CCE preparations were performed at 60°C outlet air temperature to maximize the possibility for inactivation of the enzyme as per the previous experimental results; the differences in the retention of β-gal activity, if any, would likely be more noticeable than if lower temperatures were employed. All sugar-containing CCE preparations were also freeze-dried. The results presented in Figure 8-3 show that 5% (w/w) lactose in CCE preparation caused significantly ($p<0.05$) higher retention of activity in comparison to the other two sugars at the same concentrations during spray drying. However, addition of 10% sucrose improved the preservation of β-gal activity, resulting in over 80% retention which was similar to that achieved by lactose. The addition of cellobiose also achieved significantly

($p<0.05$) higher preservation of β-gal activity in comparison to the control CCE, but the activity retained was significantly ($p<0.05$) lower than with the other two sugars.

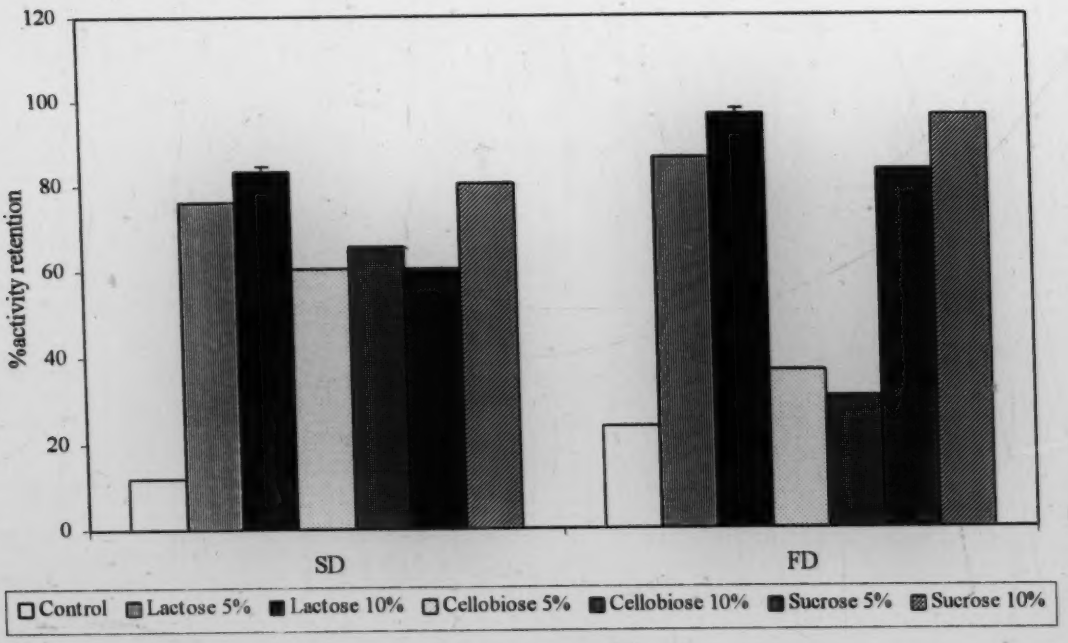

Figure 8-3: The β-galactosidase activity retention in CCE powders from *Lb. bulgaricus* 11842 prepared with the addition of different sugars upon spray drying at 60°C outlet air temperature or freeze drying

(SD – spray drying; FD – freeze drying; control – CCE in skim milk salt buffer; lactose, sucrose and cellobiose, 5 and 10% (w/v) solutions in SMSB; Bars present the adjusted standard error of the mean - ±1.28)

Likewise, protection by cellobiose upon freeze-drying was much lower than that obtained by addition of lactose or sucrose, which resulted in nearly identical outcomes, retaining almost all of the β-gal activity when 10% (w/w) of either sugar was added to the CCE.

8.3.3. Stability of CCE powders during storage

The stability of the enzyme activity was significantly ($p<0.001$) correlated to water activity of the dry powders. The most significant changes in the retention of β-gal activity during storage in CCE powders obtained by spray- or freeze-drying are presented in Table 8-3. Processing at lower outlet air temperatures (40 and 50°C) resulted in powders with a higher moisture content and thus a higher water activity (Table 8-4; the similar data for intermediate concentrations is shown in Appendix, Table A-2); the samples with higher a_w experienced a significant ($p<0.05$) loss of β-gal activity, in some cases as soon as 15 days of storage. The control sample obtained after drying at 40°C was affected the most, losing over 30% of the initial activity after 30 days of storage. Noticeably, the retention of enzyme activity was affected to a much lesser degree in the CCE powders containing whey protein preparations. Also, all CCE samples processed at 60°C showed little loss of the β-gal activity up to 30 days of storage.

Generally, freeze-dried CCE powders were more stable than those spray-dried at 40 or 50°C, with the control sample the only one which showed a significant ($p<0.05$) loss of the β-gal activity after 30 days of storage (Table 8-3). All freeze-dried samples had a much lower moisture content and corresponding a_w than the spray-dried counterparts. The storage conditions resulted in significant ($p<0.05$) increases in moisture content and a_w of all spray and freeze dried CCE powders (Table 8-4).

Table 8-3: Statistically significant changes in the β-galactosidase activity after 15 and 30 days of cold storage at 7°C of dry CCE powders from *Lb. bulgaricus* 11842 prepared with the addition of different concentrations of drying adjuncts by spray drying at 40, 50 or 60°C air outlet temperature or freeze-drying

Treatments*	Activity retention, % 15 days	p	Activity retention, % 30 days	p
SD 40				
Control	83.4	<0.0001	66.3	<0.0001
Lac 5	93.4	<0.001	91.3	<0.001
SM 10	90.7	<0.001	86.6	<0.0001
SM 30	NS*	>0.05	88.4	<0.0001
WPC 10	NS	>0.05	93.0	<0.005
WPC 15	NS	>0.05	91.4	<0.001
WPI 5	NS	>0.05	94.9	<0.05
WPI 10	NS	>0.05	94.0	<0.05
SD 50				
Control	96.0	<0.001	88.8	<0.001
Lac 10	NS	>0.05	93.3	<0.001
SM 10	NS	>0.05	89.2	<0.001
SM 20	NS	>0.05	92.2	<0.01
SM 30	NS	<0.001	82.4	<0.001
WPC 15	NS	>0.05	94.0	<0.05
WPI 5	NS	>0.05	95.1	<0.05
SD 60				
Control	92.1	<0.001	88.2	<0.001
SM30	94.7	<0.05	83.8	<0.001
FD				
Control	94.9	<0.01	88.8	<0.0001

***Control – CCE in skim milk salt buffer (SMSB); Lac 5, 10, 15; WPC 5, 10, 15; WPI 5, 10, 15 – final lactose, WPC and WPI concentration (w/w) in SMSB; SM 10, 20, 30 – final skim milk solids concentration (w/v) in deionized water; SD – 40, 50 and 60 – spray drying and corresponding outlet temperatures; NS – no statistically significant loss of activity, p>0.05.**

Table 8-4: Moisture content and water activity of selected dry powders containing crude β-galactosidase extracts from *Lb. bulgaricus* 11842

Treatment*	Moisture content, %			Water activity	
	Initial	15 days	30 days	Initial	30 days
SD 40					
C	6.5	10.6	13.7	0.311	0.491
Lac - 5	5.6	8.3	11.2	0.342	0.463
Lac - 15	4.9	5.7	8.1	0.316	0.401
SM - 10	5.5	6.4	7.3	0.355	0.431
SM - 30	5.5	6.1	6.8	0.329	0.397
WPC - 5	5.0	6.9	8.2	0.212	0.399
WPC - 15	4.9	5.9	7.4	0.196	0.296
WPI - 5	5.1	6.5	7.2	0.219	0.319
WPI - 15	4.6	5.4	6.1	0.250	0.350
SD 50					
C	6.8	8.3	10.4	0.280	0.410
Lac - 5	6.5	7.0	7.2	0.297	0.357
Lac - 15	3.9	4.6	5.5	0.278	0.338
SM - 10	5.2	6.0	6.8	0.332	0.392
SM - 30	5.1	5.6	6.0	0.291	0.351
WPC - 5	4.3	6.1	7.3	0.216	0.306
WPC - 15	3.4	5.2	6.4	0.199	0.289
WPI - 5	4.3	4.9	5.5	0.222	0.312
WPI - 15	3.7	4.0	4.2	0.204	0.294
SD 60					
C	4.6	7.6	11.4	0.256	0.465
Lac - 5	4.0	6.9	9.4	0.308	0.368
Lac - 15	2.7	3.7	4.4	0.256	0.316
SM - 10	3.3	4.6	5.9	0.255	0.315
SM - 30	4.2	4.8	5.0	0.275	0.335
WPC - 5	4.3	5.9	7.3	0.226	0.296
WPC - 15	2.9	4.8	6.4	0.207	0.277
WPI - 5	4.0	4.5	4.9	0.216	0.286
WPI - 15	3.6	4.3	5.2	0.215	0.285
FD					
C	1.9	3.4	5.6	0.260	0.404
Lac - 5	1.0	3.9	8.4	0.193	0.248
Lac - 15	5.5	6.3	7.5	0.319	0.374
SM - 10	1.6	4.7	6.5	0.171	0.221
SM - 30	1.8	3.6	4.6	0.125	0.175
WPC - 5	1.3	2.2	3.6	0.043	0.246
WPC - 15	0.4	1.7	3.3	0.033	0.163
WPI - 5	0.4	2.2	3.6	0.035	0.309
WPI - 15	0.4	2.1	3.4	0.038	0.237
SEM	0.41			0.016	

***SD and FD – spray and freeze drying, respectively; Lac, SM, WPC and WPI – lactose, skim milk total solids, whey protein concentrate and isolate in indicated concentrations (w/w); SEM – standard error of the mean.**

8.4. Discussion

The presence of lactose in all CCE preparations had a substantial effect on the preservation of β-gal activity upon drying. All additions of dairy materials as drying adjuncts resulted in considerably higher preservation of enzyme activity in comparison to control preparation, but the best protection was offered by lactose alone or by skim milk containing similar lactose concentrations. The addition of whey protein products, although resulting in some preservation of enzyme activity in comparison to the control, had a much less pronounced effect, likely due to the much lower lactose content. WPC provided slightly better enzyme protection in comparison to WPI, again possibly due to the higher lactose content in the WPC powder (Table 8-1).

The addition of whey proteins to CCE preparations substantially improved the retention of β-gal activity in freeze-dried powders in comparison to the spray dried ones. The protective mechanism remains unknown, although Anchordoquy et al. (2001) showed that certain polymers may stabilize some enzymes, maintaining the protein quaternary structure during freeze-drying. But little is known about the conformational structure of β-gal from *Lb. bulgaricus* 11842. Schmidt et al. (1989) sequenced the β-gal gene from *Lb. bulgaricus* B131 and hinted that this enzyme may not have a tetrameric organization.

All sugar-containing CCE preparations resulted in substantially higher β-gal activity in the dry powders in comparison to the control CCE. However, the nature of the sugars added had a profound effect on the preservation of enzyme activity. Upon dehydration, the enzymes may undergo conformational changes resulting in the loss of activity (Prestrelski et al., 1993). Such changes may be prevented by addition of sugar

through either water replacement (Carpenter and Crowe, 1988) or vitrification due to the physical entrapment of the enzyme into an amorphous sugar matrix (Franks et al., 1991).

The dilution effect (Costantino et al., 1994) may prevent the aggregation of β-gal, which may be another cause of the activity loss (Yoshioka et al, 1993). From our results, it is not evident which, if any, of these mechanisms could be primarily responsible for the stabilization of β-gal by sugars. The absence of the competitive inhibition by cellobiose during the lactose hydrolysis as ascertained during the confirmatory experiments might be an indication of the inability of cellobiose to attach to the enzyme active center. This, in turn, might have resulted in the poor protective effect of cellobiose.

Substantially better preservation of enzyme activity was obtained with 5% lactose in spray dried powders in comparison to the other sugars might have been due to enzyme-substrate binding, which consequently might have preserved the active center conformation of the enzyme, resulting in the higher preservation of enzyme activity. The difference in the retention of β-gal activity also might have been influenced by the amorphous/crystalline state of the investigated sugars. Millqvist-Fureby et al. (1999a,b) showed that lactose readily attained the amorphous form regardless of the drying method, while sucrose acquired a varying degree of crystallinity. Possibly, at 5% sucrose concentration, the content of the amorphous fraction was not enough to prevent the enzyme deactivation resulting in substantially lower β-gal activity in comparison to lactose at the same concentration. Increasing sucrose concentration to 10% would have resulted in higher content of the amorphous segment, possibly explaining the higher enzyme activity preservation which was similar to that achieved by the lactose at equal concentration. Lower retention of β-gal activity in spray dried CCE powders containing

sugar in comparison to freeze-dried powders might have been caused by the surface denaturation of the enzyme or the concentration of salts during the drying process. Proteins tend to accumulate on the droplet surface, making them more susceptible to detrimental effects of heat (Fäldt and Bergenståhl, 1994; Millqvist-Fureby et al., 1999a).

The stability of dry enzyme preparations is strongly affected by storage conditions and physical properties of the protective compounds (Sun and Davidson, 1998). Enzymes are stabilized by the amorphous form of sugars, which may start to crystallize resulting in the loss of the enzyme activity upon prolonged storage (Suzuki et al., 1997; Millqvist-Fureby et al., 1999a,b). This could be one of the reasons for the observed slight but statistically significant ($p<0.05$) decrease of retention of β-gal activity in dry CCE powders containing high concentrations of lactose. WPC and WPI CCE powders containing less lactose than buffered lactose or skim milk CCE preparations resulted in better enzyme stability retention during storage in comparison to other examined powders. Dry CCE powders containing whey proteins retained on average more than 95% of the initial β-gal activity after 30 days of storage. However, the initial enzyme activity retention immediately after drying was lower than that obtained with lactose or skim milk.

A substantial loss (over 30%) of the enzyme activity in the 40°C spray dried control powder containing no lactose over 30 day storage period might have been caused by completely different factors such as high water activity (Labuza, 1980; Rockland and Nishi, 1980). Similarly, high initial moisture content and consequently water activity, accompanied with the high moisture uptake during storage of the low temperature (40 or 50°C) spray dried powders, resulted in the loss of β-gal activity ranging from 5 to 16% in

comparison to preparations obtained at the higher temperature (60°C). The initial water activity in the spray dried powders prepared at low temperature might have been sufficiently high to allow deteriorative reactions such as oxidation and/or activity of proteolytic enzymes to proceed. Some deteriorative enzymes are known to remain active in dry foods at intermediate and low water activity, especially if the drying temperature was low (Schwimmer, 1980). The low processing temperatures of the CCE preparations, containing a complex mixture of intracellular enzymes originating from a thermophilic bacterium, may have resulted in the preservation of undesirable proteolytic enzymes, which could have caused the β-gal deactivation. Alternatively, Yoshioka et al. (1992) suggested that the β-gal inactivation in freeze-dried powders containing different moisture levels might occur due to protein aggregation via covalent interactions.

It appears that the presence of lactose was essential for almost complete preservation of the β-galactosidase activity in CCE preparations upon drying through one or several mechanisms encountered. However, lactose was somewhat less effective than whey proteins in preserving the residual enzyme activity during storage, possibly due to recrystallization and/or higher initial a_w. The addition of whey proteins also provided appreciable β-gal activity retention during freeze-drying, a phenomenon still awaiting proper elucidation.

8.5. Conclusions

The addition of dairy-based drying adjuncts (lactose, skim milk, whey protein products) to CCE preparations from *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 had a major effect on the preservation of the β-gal activity during spray and

freeze-drying. At a low spray drying outlet air temperature (40°C) all adjuncts in concentrations higher than 5% provided almost complete protection of β-gal activity. However, only CCE powders containing lactose retained substantial β-gal activity at 60°C outlet air temperature. Similarly, lactose-containing adjuncts showed a substantially better protection in the enzyme preservation during freeze-drying at low initial total solids concentrations (5 and 10%) than those with little lactose present. However, the retention of enzyme activity improved with increased concentration and no substantial difference in the activity in freeze-dried CCE powders was observed at high adjunct concentrations. The mechanism of the protection of β-gal activity by lactose could not be fully explained. Cellobiose, as a lactose analog, preserved the enzyme activity poorly. Lactose exerted better enzyme protection than sucrose or cellobiose at 5% concentration, indicating that the protection of the enzyme active center conformation might be one of the reasons for the overall β-gal stability. However, no significant difference ($p>0.05$) in the enzyme preservation was observed between lactose and sucrose at 10% concentration. Although lower outlet air temperatures during spray drying resulted in higher retention of β-gal activity than at higher temperatures, higher moisture content and subsequent increase in water activity during storage caused faster enzyme activity loss. Possibly the lactose crystallization, formation of enzyme agglomerates and/or proteolytic activity in dry CCE powders were some reasons for the β-gal deactivation. Drying preservation of CCEs with high β-gal activity appears possible when drying adjuncts containing lactose are used and the drying results in a very low final a_w of the dried powder.

8.6. References

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Chapter 9*

Sensory Effects of Lactose Hydrolysis in Milk by Crude Cellular Extracts from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842

9.1. Introduction

An enzymatic approach to lactose hydrolysis has been considered as one of the most applicable methods for lactose conversion in the dairy industry. The enhanced sweetness and solubility of lactose-hydrolyzed preparations may also expand the possibilities for the whey utilization (Zadow, 1993; Mahoney, 1997), diminish some of the technological problems associated with the lactose crystallization (Patocka and Jelen, 1988) or alleviate the problems associated with lactose malabsorption and intolerance affecting 70% of the world's population (Lee and Krasinski, 1998). Another possible advantage of enzymatic hydrolysis of lactose is the production of galactooligosaccharides, a bifidogenic factor, resulting in enhancement of health promoting properties (Gallaher, 1999).

Recently, a process for lactose hydrolysis by β-galactosidase (β-gal) in the form of the crude cellular extract (CCE) obtained by mechanical disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 has been proposed (Vasiljevic and Jelen, 2001) and the hydrolytic effectiveness has been demonstrated (Kreft and Jelen, 2000; Kreft et al., 2001; Vasiljevic and Jelen, 2002). Depending on the environmental conditions during lactose hydrolysis, β-gal preparations also exerted a certain degree of proteolytic activity as well as the growth of the remaining viable cells (Vasiljevic and Jelen, 2002), which in turn

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could adversely affect the sensory characteristics of a lactose-hydrolyzed product. The sensory properties could also be influenced by the processing conditions during the culture fermentation, such as the selection of the neutralizer used for pH maintenance.

The objective of the present study was to characterize the sensory impact of lactose hydrolysis in skim milk by crude β-gal preparations from *Lactobacillus bulgaricus* 11842 with the emphasis on identification of the imparted off-flavors.

9.2. Materials and methods

9.2.1. Culture cultivation, cell collection and CCE preparation

Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842 (Lb. 11842) was obtained from the Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada. The culture production was carried out as reported previously (Vasiljevic and Jelen, 2002) by 10 hour fermentations in 2L batches of pre-sterilized commercial skim milk (Lucerne milk processing plant, Edmonton, AB) at 43°C and pH 5.6 maintained by three different neutralizers: ammonium, potassium or sodium hydroxide. Upon termination, the fermented skim milk was cooled and cells were gathered by centrifugation (Beckman model J2-21, Beckman Coulter Inc, Fullerton, CA) at 14000 x g and 4°C for 10 min. The collected cell paste batches were frozen and stored until required. When needed, a frozen batch (approx. 14% dry matter) was thawed at 37°C in a water bath and approximately 80 mL was transferred into a bead mill (Dyno-Mill type KDL, Impandex Inc., Maywood, NJ). The disruption was performed using 0.2 to 0.3 mm glass beads for 2 min at 4°C. The disrupted material, termed crude cellular

extract (CCE), was washed with the appropriate amount of commercial pasteurized skim milk (Lucerne) to obtain the desired concentrations of CCE in the milk to be hydrolyzed.

9.2.2. Lactose hydrolysis procedure

For the expert sensory evaluation, the samples contained 2 or 4% CCEs in the skim milk and the lactose hydrolysis was performed at 7 or 20°C for 24 hrs. The samples were served to the panelists at the temperature of the hydrolysis.

The lactose hydrolyzed skim milk samples for the second sensory analysis were prepared with 1 or 2% (v/v) of CCEs; the mixtures were held in water bath at 55°C with mixing for approximately 6 hours, till 70 to 75% conversion was achieved as determined by cryoscopy (Vasiljevic and Jelen, 2002). Lactose hydrolyzed milk samples were cooled to 7°C, held overnight and served cold to the panelists the following day. The lactose hydrolyzed chocolate milk samples were prepared by addition of cocoa powder (1.6% w/v) and sucrose to lactose-hydrolyzed skim milk previously prepared using 1% sodium- or potassium CCE addition. The amount of sucrose added, along with the products of lactose hydrolysis, resulted in the approximate sweetness intensity equal to that of 6% sucrose solution. The control was prepared from untreated skim milk, 1.6% (w/w) cocoa powder and 6% (w/w) sucrose. All chocolate milk samples were passed through microfluidizer (model M-110EH, Microfluidics, Newton, MA) with two chambers: H10Z (100μ) and F20Y (75μ), serially connected, at 172.4 MPa to enhance the particle distribution and stabilize the suspension; cooled to 7°C; and served to the panelists. A commercial β-galactosidase preparation (Lactaid, McNeil Nutritionals, Ft. Washington, PA) was used to produce lactose hydrolyzed skim milk control samples; as per the

manufacturer's instructions, the preparation was used at 37°C. The regular commercial skim milk served as an unhydrolyzed control.

9.2.3. Sensory evaluation

The sensory protocol was approved by the Human Research Ethics Board of the Faculty of Agriculture, Forestry and Home Economics at the University of Alberta. The expert taste evaluation was performed in order to determine the suitability of CCE preparations for the main sensory assessment. The initial assessment was conducted independently, by three experienced dairy product evaluators. They were instructed to note all major off-flavors in lactose hydrolyzed skim milk samples and assign a partial score based on the degree of difference from a control reference sample. The grading scale from 0 to 4 was used during evaluation, with 0 presenting no difference and 4 being extremely different from the unhydrolyzed control sample. A consensus value was reached by discussion.

The second sensory analysis was conducted with an untrained panel (n=10) to characterize the effect of selected neutralizers used during the production of CCEs on sensory characteristics of lactose-hydrolyzed products. Panelists were selected from the faculty, staff and student population of the University of Alberta, Department of Agricultural, Food and Nutritional Science. The panel was composed of 6 female and 4 male individuals. Panelists were instructed to record the intensity of sweetness on a 150 mm scale and to list any off flavor in the skim milk, assumed to be caused by the neutralizers used for the production of CCE and/or by the side reactions during the lactose hydrolysis. Samples (50 mL) in transparent plastic cups, labeled with 3 digit

codes were presented in random order under red fluorescent lights in the sensory testing facility. Panelists cleansed their palates between samples with distilled water and unsalted soda crackers. The results were analyzed measuring the distances of marks (in mm) denoting the lactose-hydrolyzed samples from the unhydrolyzed control (Gabrielsen, 2000). The same panelists were also used to evaluate lactose hydrolyzed chocolate milk samples by ranking them in order of preference.

9.2.4. Statistical analysis

Statistical significance of differences was determined by ANOVA and Tukey's test to separate the means where appropriate using the SAS statistical software (SAS Institute, 1992). Unless indicated otherwise, results are expressed as average ± standard error of the mean (SEM) using all available data. The ranking results were processed using the Friedman ranking test. The level of significance was set at $p=0.05$.

9.3. Results

9.3.1 Expert assessors

The first assessment by three experienced dairy products evaluators was aimed to establish a general suitability of CCE preparations for further sensory evaluation. The results of this evaluation are presented in Table 9-1. The concentration of CCEs and temperature at which lactose hydrolysis and sensory analysis were conducted had an apparent effect. Low temperature treated lactose hydrolyzed samples generally received lower marks (less difference) than those processed at higher temperature. The addition of

4% (v/v) CCEs to skim milk resulted in substantially increased off-flavors in comparison to 2% (v/v) noted immediately after addition. All CCE treated samples were characterized by off-flavors specific to neutralizers employed during the culture propagation such as acidity and saltiness with the intensity depending on the CCE concentration.

Table 9-1: Expert sensory assessment* of the lactose-hydrolyzed skim milk prepared by the addition of 2 or 4% CCEs obtained by cultivation of *Lb. bulgaricus* 11842 using ammonium, sodium or potassium hydroxide as neutralizer

Sample**	0h	Remark	24h	Remark
Control	0	Clean	0	Clean
NH_4 - 2C	2	Slightly acidic/ salty/bitter/unclean	2+	Somewhat sweet/ acidic/salty/bitter
NH_4 - 4C	2+	Acidic/extremely salty/unclean	3	Somewhat sweet/ acidic/salty
NH_4 - 2R	3+	Slightly acidic/ very salty/bitter/ grossly unclean	4	Somewhat sweet/ very acidic/ salty/unclean
NH_4 - 4R	4	Acidic/extremely salty/bitter/ grossly unclean	4+	Slightly sweet/very acidic/salty/bitter/ unclean
Na - 2C	1	Slightly salty	2	Sweet/slightly salty
Na - 4C	1.5	Salty	3	Very sweet/salty
Na - 2R	2+	Salty	3+	Sweet/slightly salty
Na - 4R	3+	Very salty	4+	Sweet/salty
K - 2C	0	Clean	0.5	Slightly sweet/Clean
K - 4C	1	Slight unidentified off-flavors	3	Very sweet/slight off-flavors
K - 2R	2	Clean/some off flavors	3	Sweet/slight off flavors
K - 4R	2	Slight unidentified off-flavors	3+	Very sweet/slight off flavors

***Scores: 0 – no difference, 4 – extreme difference from reference control; **Control – commercial pasteurized skim milk; NH_4, Na, K – ammonium, sodium and potassium hydroxide-produced CCE; 2, 4–the CCE concentration (w/v); C, R–cold and room temperature, 7 and 20°C, respectively; n = 3.**

Skim milk treated with ammonium CCE was noticeably more acidic, salty and unpleasantly bitter in comparison to the other two CCEs at the beginning of lactose hydrolysis. The acidity increased by the end of the hydrolysis, which might have been caused by the growth of the viable cell remaining in this CCE after disruption. Based on these findings, the CCE from NH_4 fermentation was judged unsuitable for further sensory trials because of grossly detrimental sensory effects, although the use of NH_4OH during fermentation resulted in improved growth characteristics and enhanced β-gal activity (Vasiljevic and Jelen, 2002). Sodium-produced CCE also resulted in increased saltiness in lactose-hydrolyzed samples after immediate addition, which, however, was masked to some degree by increased sweetness at the end of the lactose hydrolysis.

9.3.2. Untrained taste panel

The second sensory evaluation of the lactose-hydrolyzed skim milk was performed with an untrained panel in order to evaluate the impact of the best two neutralizers used for the preparation of the CCEs. The sensory evaluation results for the samples prepared with the sodium and potassium CCE are presented in Table 9-2, where a lower value represents a lower degree of attribute intensity. The targeted degree of hydrolysis was set between 70 and 75%, based on the report of Mitchell and Hourigan (1993) that there was no significant ($p>0.05$) difference in determined sweetness by a sensory panel up to about 70%. Hourigan (1984) also encouraged a targeted degree of the lactose hydrolysis between 60 and 70% as being sufficient to reduce clinical symptoms in lactose maldigesters. Additionally, the cost associated with the degree of lactose hydrolysis over 80% would be much higher (Geilman, 1993).

The panelists were asked to characterize all lactose hydrolyzed skim milk samples in comparison to the unhydrolyzed control regarding sweetness and off-flavour intensity. The analytically determined degree of lactose hydrolysis in skim milk samples was within the targeted range (Table 9-2). Despite this, the difference in sweetness intensity between lactose hydrolyzed samples and unhydrolyzed control was significant ($p<0.05$).

Table 9-2: The means and standard errors for the sensory evaluation of lactose hydrolysis on the properties of lactose-hydrolyzed skim milk and chocolate drink preference with 1 or 2% of the Na or K based CCE

Attribute	CLP**	K - 1	K - 2	Na - 1	Na - 2	SM	SEM
Degree of lactose hydrolysis, %	75.3	71.1	74.9	72.2	75.2	0.5	0.43
Sweetness intensity*	40^{b}	43^{b}	40^{b}	54^{b}	37^{b}	0^{a}	8.45
Off-flavor intensity*	93^{c}	11^{a}	70^{c}	31^{bc}	59^{c}	0^{a}	9.57
Chocolate drink preference	48^{b}	27^{a}	32^{ab}	28^{a}	50^{b}	25^{a}	

[a, b, c] - values in the same row with different letters are significantly different ($p<0.05$); *values (in mm) indicate degree of difference from reference control on a linear scale. ** CLP – commercial β-galactosidase preparation; Na – 1,2 – 1 and 2% addition of NaOH CCE; K – 1,2 – 1 and 2% addition of KOH CCE; SM – commercial lactose unhydrolyzed skim milk or chocolate milk preparation. SEM – adjusted standard error of the mean; average values, n = 10.

The addition of 2% CCEs resulted in significant ($p<0.05$) impact on the perception of off-flavors. The lactose-hydrolyzed skim milk prepared using the commercially available β-galactosidase preparation surprisingly was scored as also exhibiting significant ($p<0.05$) off-flavors due to noticeable bitterness. This could be a result of the extended exposure of the mixture to 37°C as per manufacturer's recommendations, where microbial reactions including proteolysis could have been at

optimum. The major off-flavor characteristics for the lactose hydrolyzed skim milk samples obtained with the addition of 2% CCEs produced with both Na and K were saltiness and bitterness, presumably imparted by the salts present in the mixture originating from the neutralization in the culture propagation step. The intensity of the off-flavor in lactose hydrolyzed skim milk samples obtained using the 1% (v/v) CCE produced with KOH was not significantly ($p>0.05$) different from that of regular unhydrolyzed skim milk.

The lactose hydrolyzed chocolate milk samples were prepared by adding the cocoa powder and sucrose to previously lactose-hydrolyzed skim milk in the amount to match sweetness intensity of regular chocolate milk. The addition slightly improved the overall acceptance of the lactose-hydrolyzed products and no significant ($p>0.05$) difference was observed between the control and lactose hydrolyzed samples obtained with 1% CCEs or 2% KOH CCE addition (Table 9-2). Again, the lactose hydrolyzed chocolate milk prepared with the addition of commercial β-gal preparation and 2% NaOH CCE were ranked the lowest.

9.4. Conclusions

The use of CCEs at high concentrations (2 or 4%) resulted in substantial off-flavors even after immediate addition as determined by the expert sensory evaluators. The increase in the off-flavor intensity at the end of the lactose hydrolysis for sodium- or potassium-produced CCE was due to noticeable sweetness. In contrast, the perceptible acidity, saltiness and bitterness in the lactose hydrolyzed samples obtained using ammonium hydroxide as neutralizer resulted in grossly detrimental sensory effects. In

sensory evaluations of the products obtained with Na and K produced CCEs by untrained assessors, all lactose-hydrolyzed samples were significantly ($p<0.05$) sweeter than the unhydrolyzed control. However, only addition of 1% (v/v) potassium CCE resulted in no significant difference ($p>0.05$) in off-flavor intensity in comparison to the control. The higher concentration imparted undesirable saltiness and bitterness in lactose hydrolyzed products. The lactose hydrolyzed chocolate milk samples prepared with additional sucrose and 1% CCE were acceptable and no significant difference ($p>0.05$) was observed in comparison to the unhydrolyzed control. Since skim milk is one of the most sensitive dairy products regarding sensory defect development and identification, lactose hydrolysis in dairy products appears possible when CCEs are added at low concentrations even without use of masking flavors.

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Chapter 10

Conclusions and recommendations for further research

10.1. Conclusions

Several reasons, such as abundance of whey and prevalence of lactose intolerance, have been driving industrial and scientific attention towards lactose hydrolysis for decades. Although different approaches were proposed, only a few, mainly involving the enzyme β-galactosidase, have been commercially applied. Cost effectiveness has been indicated as one of the detriments in a number of proposed processes for the lactose hydrolysis. To minimize the cost, the methodology for the production of β-galactosidase should be economically feasible resulting in readily attainable enzyme preparations. Furthermore, the production of value added lactose hydrolyzed products may additionally minimize associated cost. This dissertation project has attempted to elaborate on previously made suggestions (Shah and Jelen, 1991; Jelen, 1993; Bury and Jelen, 2000) on a putatively cost effective application of a common dairy microorganism, *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (Lb11842), for the lactose hydrolysis in dairy products after mechanical disruption.

10.1.1. Summary of experimental results

The use of skim milk as a cultivation medium resulted in higher β-galactosidase activity in comparison to whey or whey permeate based media enriched with different whey protein preparations or MRS. Subsequent reprocessing of the cell-free skim milk

fermentation media into different commercial products could additionally minimize processing cost as well as eliminate the disposal problem associated with the use of whey in the cultivation media preparation. The selection of a neutralizer for the pH maintenance altered some of the growth characteristics of Lb11842 cultivated in skim milk under optimum conditions. Ammonium hydroxide used during culture cultivation resulted in an enhanced growth and β-galactosidase activity with concomitant suppression of the proteolytic activity of the culture when compared with sodium or potassium hydroxide. The addition of ammonium hydroxide also increased the exopolysaccharide production by the growing culture. The reasons for the enhancement of the growth characteristics of the culture remains unknown, although the ammonium utilization by Lb11842 might be one of them. Lb11842 generally produced exopolysaccharides in skim milk regardless of the neutralizer employed. The EPS forming ability of Lb11842 could be used in a range of fermented dairy products with EPS serving as a thickening agent.

The rate of the lactose hydrolysis in skim milk using β-galactosidase-containing crude cellular extract (CCE), obtained from Lb11842 cultivated in skim milk at constant pH maintained with ammonium, sodium or potassium hydroxide, was apparently affected by the CCE origin. The highest initial rate of lactose hydrolysis, obtained at 65°C, could not be maintained due to enzyme deactivation. Overall, the highest degree of the lactose hydrolysis was achieved at 55°C. Besides affecting the rate of the lactose hydrolysis, temperature influenced the growth of the residual undisrupted cells in all CCEs. While the cell growth was enhanced with concomitant pH decrease at 20 and 37°C, the cell counts were either substantially reduced at 55°C or no change was noted at 7°C. In

addition to hydrolysis of lactose, β-galactosidase-containing Lb11842 CCE exhibited transferase activity, resulting in formation of different oligosaccharides as ascertained by a HPLC determination. The rate and amount of oligosaccharides formed depended on the lactose concentration and the reaction temperature. The maximum oligosaccharide formation was achieved in 30% (w/w) lactose solution at 50°C. The lactose hydrolyzing, transferase and proteolytic activities of Lb11842 CCE were compared with β-galactosidase-containing CCEs from two other dairy cultures, *St. thermophilus* 143 (St143) and *Lb. delbrueckii* ssp. *lactis* 3078 (Lb3078). The maxima of the lactose hydrolysis and oligosaccharide production were achieved under similar conditions for all three CCEs; however, the amount of monosaccharides and oligosaccharides formed was culture dependent. Lb11842 CCE had the highest lactose hydrolyzing ability; in contrast, the most oligosaccharides were produced by St143 CCE in 30% lactose solution at 50°C. Lb11842 CCE also exhibited higher proteolytic activity than the other two studied CCEs. The maximum proteolytic activity as determined by the response surface methodology (RSM) for all three CCEs was generally reached around 43°C and low content of skim milk total solids; the proteolytic activity suppression occurred at higher concentrations of skim milk total solids (20 or 30% w/w) likely due to decrease of water activity in the system.

Two drying methods, spray drying and freeze-drying, were employed for the long-term preservation of the β-galactosidase-containing Lb11842 CCE. Regardless of the drying method used, the activity loss of β-galactosidase in the CCE preparation without any protective drying adjunct was substantial, approximating 90%. The addition of drying adjuncts, such as whey proteins and Na-caseinate at concentrations equal to

those found in milk, improved slightly the enzyme activity retention during spray drying. In contrast, during freeze-drying the whey proteins protected the enzyme substantially better, achieving approximately 30% activity retention. Over 60% preservation of the CCE β-galactosidase activity was achieved in preparations with whey or skim milk, the effect presumably caused by the presence of lactose. The temperature of the drying air during spray drying also had a substantial effect on the β-galactosidase activity retention with lower temperatures (45 and 55°C) resulting in a retention of the enzyme activity similar to that obtained by freeze-drying. On the other hand, spray drying at 65°C exit air temperature considerably reduced the β-galactosidase activity in whey and skim milk CCEs in comparison to freeze-dried preparations. Apparently, the presence of lactose in CCE preparations was crucial for the preservation of β-galactosidase activity.

In a separate drying study, effects of the addition of higher amounts of dairy-based drying adjuncts (lactose, skim milk, whey protein products) to the Lb11842 CCE preparation on the preservation of the β-gal activity were further explored. Generally, low spray drying outlet air temperature (40°C) and higher drying adjunct concentration (more than 5%) resulted in almost complete recovery of β-galactosidase activity upon reconstitution regardless of adjunct composition. However, the presence of lactose was essential for substantial enzyme activity protection at 60°C outlet air temperature during spray drying or low drying adjunct concentration (5 and 10%) during freeze-drying. The lactose protection of the β-galactosidase activity could not be explained by a simple mechanism, such as preservation of the enzyme active center, although this may have played a part in the overall enzyme stability. The β-galactosidase stability in dry powders during cold storage was influenced by the initial moisture content; higher moisture

content with concomitant increase in water activity resulted in faster enzyme activity loss.

A cursory study on the sensory impact of the lactose hydrolysis in skim milk using Lb11842 CCE was conducted to evaluate a suitability of CCEs in a sensory sensitive medium, such as skim milk, by determining any potential for off-flavor formation. Generally, the immediate CCE addition in higher concentrations (2 and 4%) resulted in considerable off-flavors as determined by experienced sensory assessors. The higher detrimental sensory effect of ammonium hydroxide-obtained CCE even resulted in elimination from further sensory studies. The untrained sensory panelists found no substantial difference in off-flavor intensity between lactose-hydrolyzed skim milk using 1% CCE produced using potassium hydroxide as a neutralizer during the culture cultivation and the unhydrolyzed control. In addition, all lactose-hydrolyzed samples were considerably sweeter than the unhydrolyzed control. The addition of chocolate and sucrose improved the overall acceptability of the lactose-hydrolyzed samples prepared by 1% CCE addition apparently by masking the off-flavors.

10.1.2. Final conclusions

This thesis project illustrated several critical indicators for the successful and feasible application of the β-galactosidase-containing CCE from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 for the lactose hydrolysis in dairy products. The media selection and culture cultivation conditions are principal factors determining the β–galactosidase activity in a crude cellular preparation. The proper selection may minimize the processing cost incurred by subsequent media reprocessing as well as affect

the sensory properties of a lactose-hydrolyzed dairy product. The choice of reaction conditions, i.e. higher temperature (50 - 60°C), during the lactose hydrolysis may not only have important ramifications on the extent of the desirable β-galactosidase activity but also prevent undesirable side reactions, such as proteolysis. The present study also showed that the spray drying could be employed for the long-term preservation of β-galactosidase in the CCE preparations with the addition of lactose-containing protective drying adjuncts.

Considering all results acquired during this study, the applicability of β-galactosidase-containing crude cellular extracts for the lactose hydrolysis in skim or other market milk products remains questionable due to undesirable sensory characteristics caused by fermentation carryovers and/or side-reactions during lactose hydrolysis. In addition, the lactose hydrolyzed skim milk is noticeably sweet, thus it may not be generally accepted by consumers as is the case with present products. On the other hand, several different areas explored in this research deserve closer attention. The enhanced exopolysaccharide formation by ammonium hydroxide addition during culture cultivation may result in feasible production of thickeners; CCE transferase activity may potentially be applied in the production of prebiotic galactooligosaccharides; and proteolytic activity of CCEs may likely be applied for the production of functional bioactive peptides. In the era of functional foods and nutraceuticals, crude cellular preparations from thermophilic dairy cultures may offer several applications more important than the β–galactosidase activity.

10.2. Recommendations for further research

As presented in the current study, the potentially economically feasible approach for the lactose hydrolysis in dairy products using mechanically disrupted thermophilic dairy cultures, termed here as the crude cellular extract (CCE), may not be the only aspect of the CCE utilization. Many different areas, related to the project, require additional research in order to elucidate and possibly utilize the properties of the growing Lb11842 culture as well as of associated CCE.

Dairy lactic acid bacteria are fastidious microorganisms, requiring numerous nutrients for growth. The appropriate media selection still appears to be an imperative for maximization of the β-galactosidase activity with economically feasible approaches, such as simultaneous production of valuable metabolites and/or subsequent media reprocessing into value-added products. Over 40 thermophilic dairy LAB species have been screened for a high β-galactosidase activity (Geciova, VSCHT Prague, personal communication), however only species used in the present study could likely be used on the industrial scale. Thus, the culture screening should be an ongoing process with simultaneous media selection. The genetical engineering of a suitable dairy microorganism, by over-expressing the β-gal gene, should be considered as a possible approach for the enhancement of β-galactosidase activity. However, the current industrial applicability of this methodology, although scientifically possible, is highly doubtful due to public perception of genetically modified organisms. The present study offered some of the alternatives for reprocessing of fermentation media after the cell collection. Additional research is required to elaborate on the development of a range of value-added products.

The substantial effect of ammonia on the growth characteristics of the Lb11842 culture still needs to be fully explained. Noticeably, the amount of exopolysaccharide (EPS) formed by the culture was also affected. However, the literature information on this effect of ammonia is lacking, although it is known that the EPS formation is influenced by carbon/nitrogen ratio in a cultivation medium (Degeest and De Vuyst, 1999). The effect of different media composition and cultivation conditions in conjunction with ammonium hydroxide, as a neutralizer for pH maintenance, on the EPS formation by different dairy starter cultures should be further investigated. The research could continue in several directions: culture and medium selection for the EPS production and isolation for applications of EPS as a thickening agent in other food systems, or *in situ* enhanced EPS production in an appropriate dairy system by a selected culture with a suitable ammonium source to prevent detrimental sensory effect in a final product. The characterization of EPS structure, as well as, rheological properties, should also be simultaneously conducted.

As shown by the sensory assessment, the use of CCE for the lactose hydrolysis appeared possible with small additions of 1% or less. The reaction conditions should be strictly monitored in order to prevent the formation of detrimental off-flavors and/or growth of the residual viable cells, remaining after mechanical disruption of the culture. Apparently the lactose hydrolysis by β-galactosidase-containing CCE also proceeds at low temperatures accompanied with a low level of CCE proteolytic activity. From the practical standpoint, a thorough investigation of the lactose hydrolysis by a selected CCE in this temperature range should be performed. The addition of a small amount of CCE accompanied by subsequent cold storage over a time period required for a desired degree

of the lactose hydrolysis (between 70 and 80%) could be carried out. The sensory assessment would be required to define any possible off-flavor formation from either carry-overs from the fermentation or proteolytic and/or other enzymatic activities and compared with that performed on lactose hydrolyzed samples achieved by CCE β-galactosidase action at high temperatures.

The established transferase and proteolytic activity of CCE could be used in several different areas with a potential industrial applicability. Galacto-oligosaccharides (GOS) formed through transferase reactions have been recognized as bifidogenic factors or prebiotics (Mödler et al., 1990). The production of GOS, from high lactose content preparations using CCE, and subsequent GOS isolation and applicability as a nutraceutical should be further evaluated. The availability of readily cell-imported peptides obtained through the action of CCE proteases on casein, a milk protein, and the presence of prebiotics, such as GOS, may result in an enhanced growth of a probiotic culture in milk and consequently better survival and higher cell viability of probiotics upon storage. Probiotics, such as *Lactobacillus acidophilus* and *Bifidobacterium* spp., grow slowly in milk due to poorly developed proteolytic systems (Shihata and Shah, 2000). The application of CCE as a dairy starter culture growth promoter has been a subject of ongoing research, conducted by another research group (Champagne, Agriculture and Agri-Food Canada, St-Hyacinthe, personal communication). The results obtained to date showed that the fermentation time required to attain desirable cell density was considerably reduced.

The focus of further research could also be directed on the exploitation of high CCE proteolytic activity. The growth of dairy starter cultures in milk depends on the

action of cell wall anchored proteases in addition to an efficient sugar import system (Juillard et al., 1995). As proposed by the present project, the cells should be disrupted for the β-galactosidase liberation. In contrast, the cell debris containing active proteases are considered as unnecessary ballast, thus a good control of processing conditions during the lactose hydrolysis in milk using CCE is required to minimize the negative proteolytic activity effect. Using membrane processes such as microfiltration, the cell debris could be physically separated from the β-galactosidase containing CCE permeate, and collected as the CCE retentate. The permeate could be applied in the lactose hydrolysis or oligosaccharide production as a protease-free CCE. The retentate, on the other hand, could be used for the casein digestion, since several peptides derived from casein possess various bioactive properties. Casomorphins, as opioid agonists (Meisel and Schlimme, 1990), and casokinins, antihyperthensive agents (Maruyama et al., 1985), are derived from α– and β-casein by digestion using food-grade enzymes, usually pepsin, trypsin and chymotrypsin (Anon, 2000). As a different approach, the action of the proteases contained in the CCE retentate on the casein could be also examined as the proteases originating from the dairy cultures have a high specificity for α- and β-casein (Juillard et al., 1995).

10.3. Closing remarks

The application of different enzyme activities (β-galactosidase, transferase, proteolysis) in the form of crude cellular preparations from thermophilic dairy starter cultures presents ample new opportunities for the dairy industry, and some of these potentials need to be evaluated carefully and completely – in particular oligosaccharide

and possibly bioactive peptides formation. While some of the results obtained by this author may have some practical importance, other areas were investigated in more basic fashion, with the uncertain technological applicability which should not be ruled out.

10.4. References

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Appendix

Supplemental data

This appendix provides the supplemental data referred to in the previous chapters.

Table A-1: The estimation of kinetic parameters in Michaelis-Menten type kinetics, k_{cat} and K_m, for the lactose hydrolysis in skim milk at different temperatures using 2% (v/v) CCE produced with three different neutralizers

Temperature °C	Na		K		NH_4	
	k_{cat}, μmol U^{-1} min^{-1}	K_m, mM	k_{cat}, μmol U^{-1} min^{-1}	K_m, mM	k_{cat}, μmol U^{-1} min^{-1}	K_m, mM
7	42.6 ±0.68	131.8 ±0.85	35.3 ±6.19	150.1 ±3.19	35.5 ±0.52	183.5 ±11.24
20	144.4 ±13.11	167.8 ±7.53	91.5 ±12.14	160.7 ±0.18	123.7 ±13.73	104.6 ±12.17
37	282.8 ±27.61	132.5 ±8.03	342.7 ±48.87	188.8 ±5.59	249.8 ±28.37	184.2 ±13.74
55	550.9 ±29.91	124.48 ±1.26	275.7 ±26.58	132.1 ±0.93	559.1 ±34.24	130.6 ±2.76

(means ± standard deviation, n = 4)

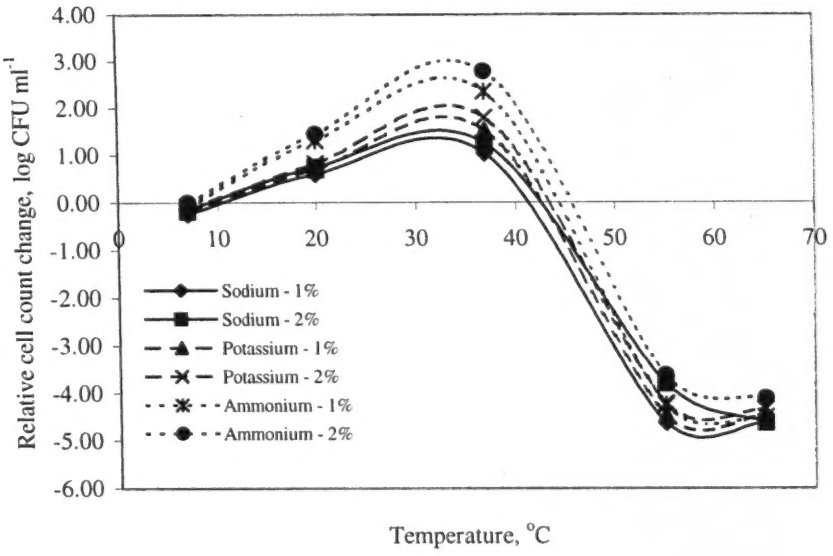

Figure A-1: Cell count change after termination of the lactose hydrolysis conducted at different temperatures in skim milk using 1 or 2% addition of sodium-, potassium- or ammonium-produced CCE from *Lb. bulgaricus* 11842

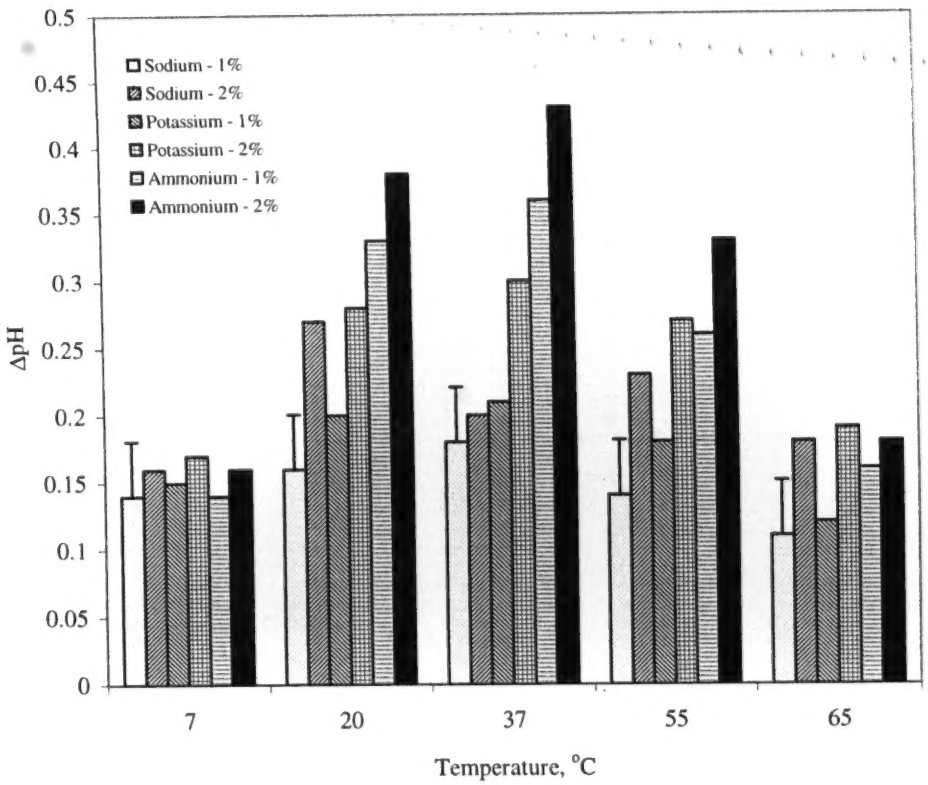

Figure A-2: Relative pH change after termination of the lactose hydrolysis performed at different temperatures in skim milk using 1 or 2% addition of sodium- potassium- or ammonium-produced CCE from *Lb. bulgaricus* 11842

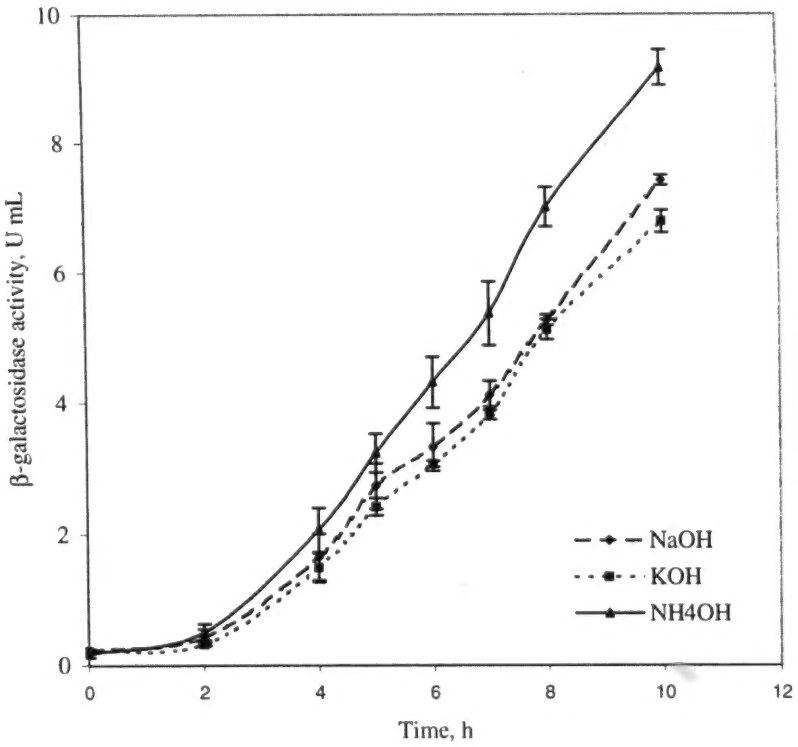

Figure A-3: The β-galactosidase activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 cultivated in skim milk at constant pH 5.6 maintained by NaOH, KOH or NH_4OH as neutralizers as determined by ONPG test

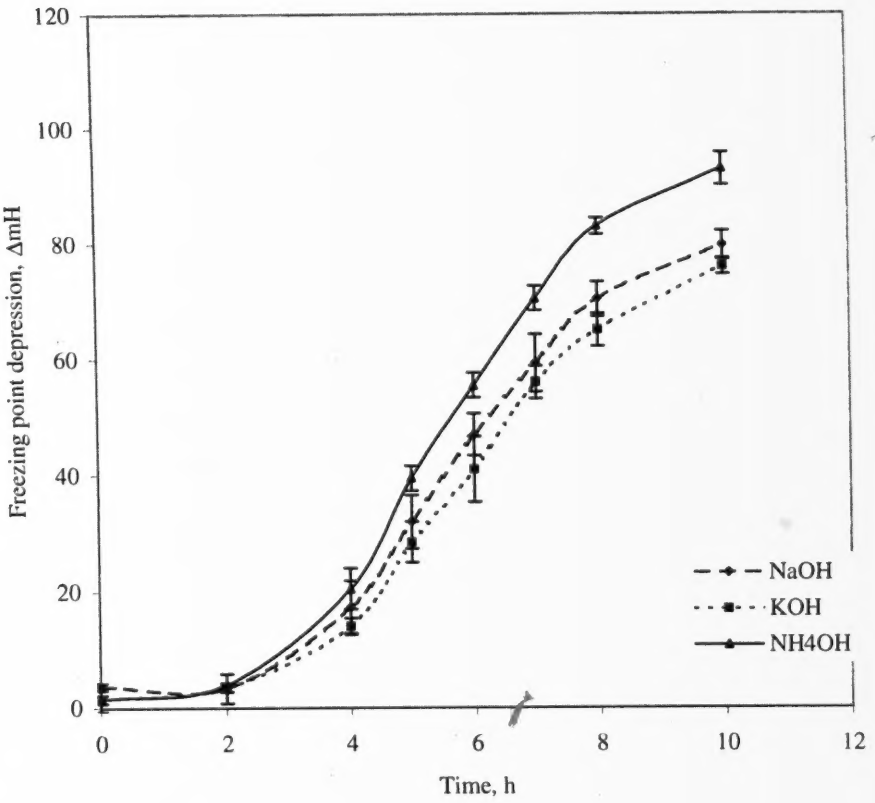

Figure A-4: The β-galactosidase activity (as determined by cryoscopy) of *Lb. bulgaricus* ATCC 11842 cultivated in skim milk at constant pH 5.6 maintained by NaOH, KOH or NH_4OH as neutralizers

Table A-2: Water activity of the cell-free spent fermentation media after termination of fermentation by *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 cultivated in skim milk at pH 5.6 maintained by NaOH, KOH or NH_4OH
(SEM – adjusted standard error of the mean; R^2 – coefficient of determination)

Neutralizer	Water activity, a_w	
	0 hr	10 hr
NaOH	0.979[a]	0.974[a]
KOH	0.979[a]	0.975[a]
NH_4OH	0.979[a]	0.976[a]
SEM	0.001	0.001
R^2	0.94	0.94

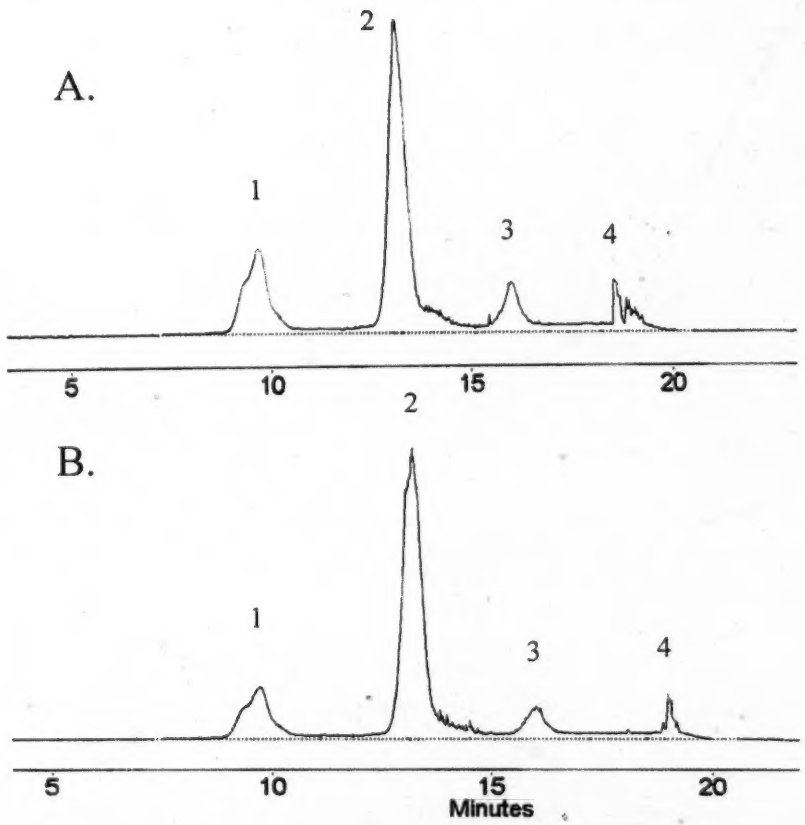

Figure A-5: Typical HPLC chromatograms obtained analyzing products of the lactose hydrolysis by A) St143 or B) Lb3078 CCE preparation as acquired by Jordi oligosaccharide column

(Peaks identified as follows: 1 – monosaccharides; 2 – disaccharides; 3 – trisaccharides; 4 – tetrasaccharides)

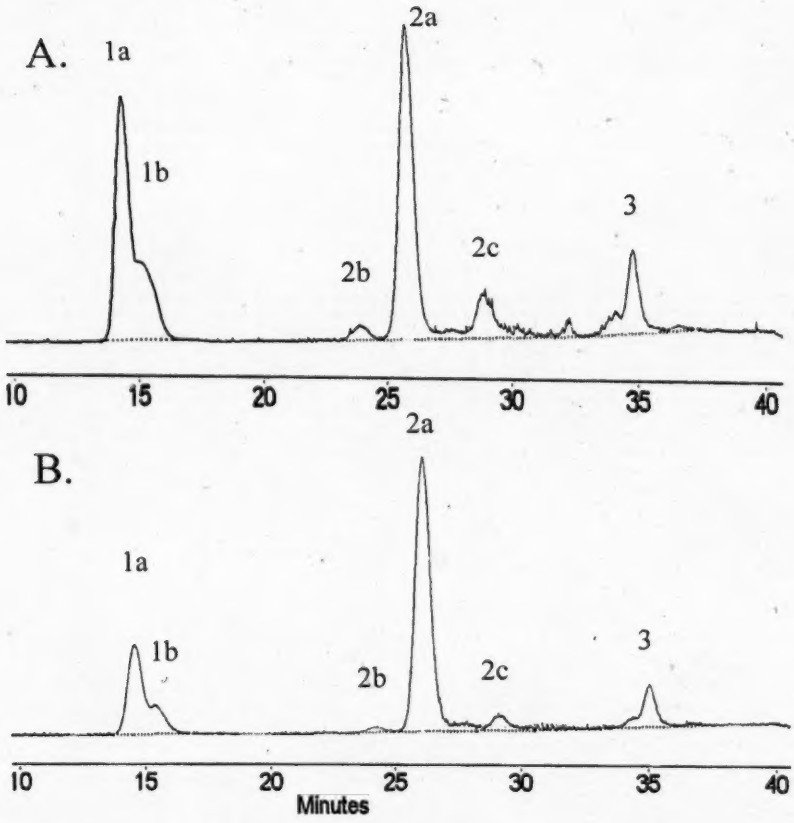

Figure A-6: Typical HPLC chromatograms obtained analyzing products of the lactose hydrolysis by A) St143 or B) Lb3078 CCE preparation as acquired by Supelcosil carbohydrate column

(Peaks identified as follows: 1a – glucose; 1b – galactose; 2a – lactose; 2b and 2c – unidentified disaccharides; 3 - trisaccharides)

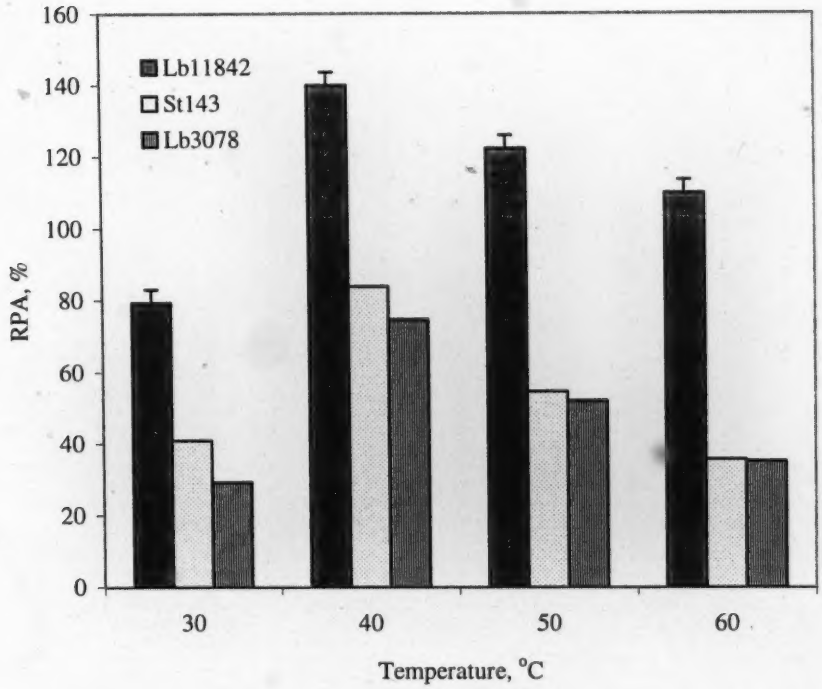

Figure A-7: Relative proteolytic activity (RPA, %) of the β-gal-containing CCEs from three thermophilic dairy cultures during the lactose hydrolysis in 20% (w/w) skim milk preparation performed at different temperatures.
(Lb11842 – *Lb. delbrueckii* ssp. *bulgaricus 11842*; St143 – *St. thermophilus* 143; Lb3078 – *Lb. delbrueckii* ssp. *lactis* 3078; bars present adjusted standard error of the means; n=4 or more)

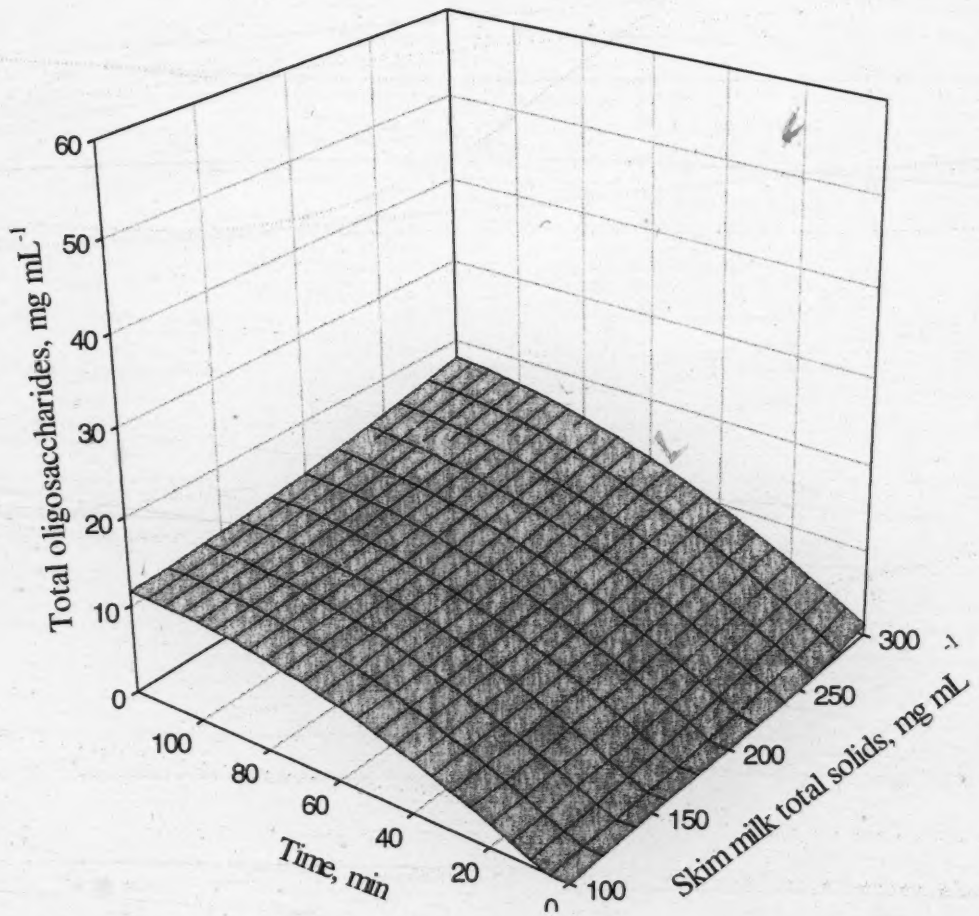

Figure A-8: Predicted oligosaccharide formation during the lactose hydrolysis in buffered lactose solutions by *Streptococcus thermophilus* 143 CCE at 50°C

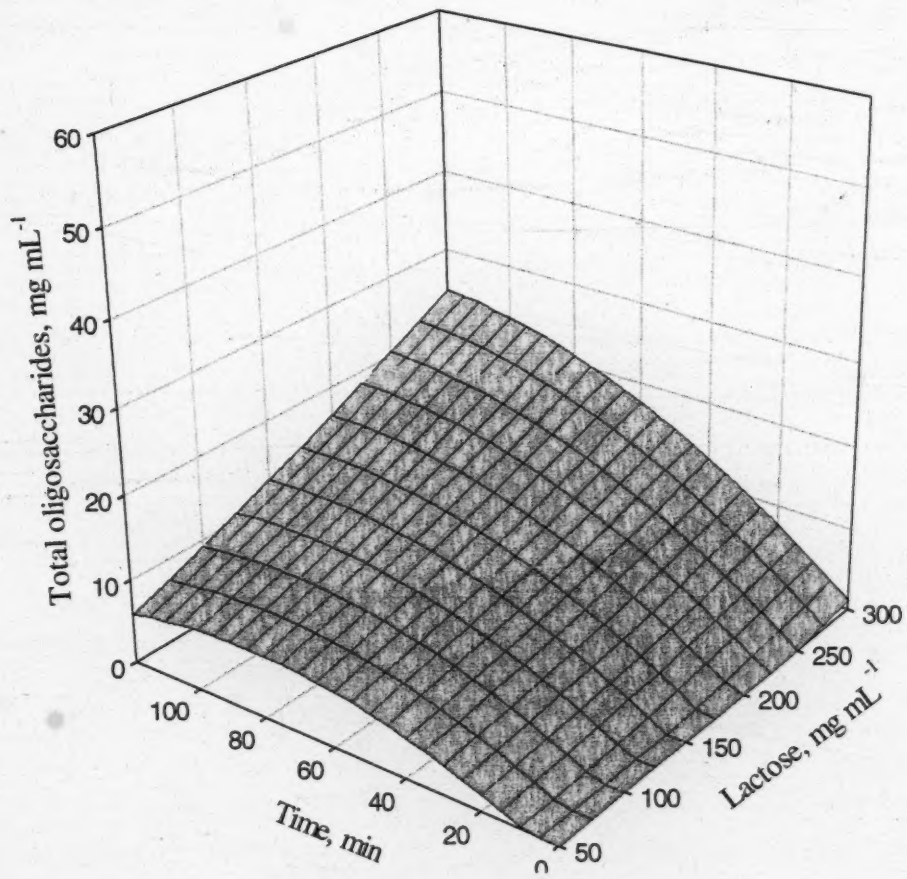

Figure A-9: Predicted oligosaccharide formation during the lactose hydrolysis in buffered lactose solutions by *Lactobacillus delbrueckii* ssp. *lactis* 3078 CCE at 50°C

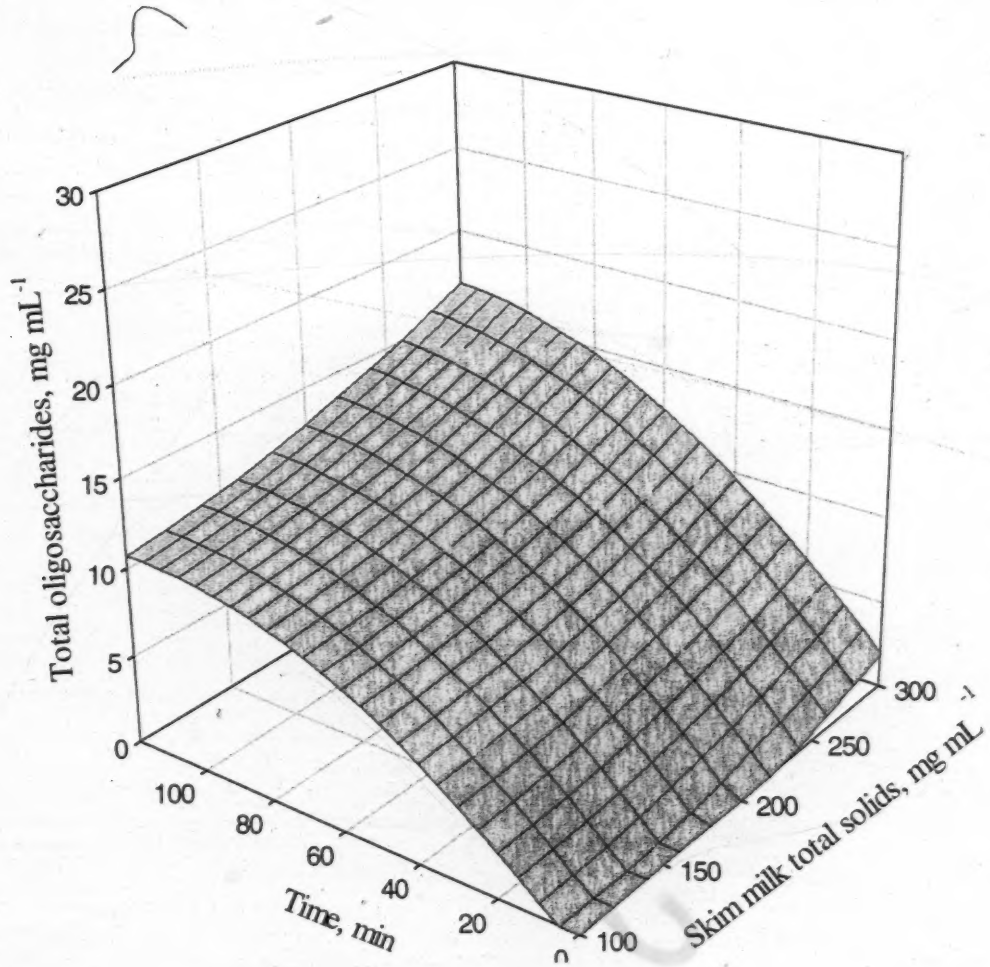

Figure A-10: Predicted oligosaccharide formation during the lactose hydrolysis in skim milk systems by *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 CCE at 50°C

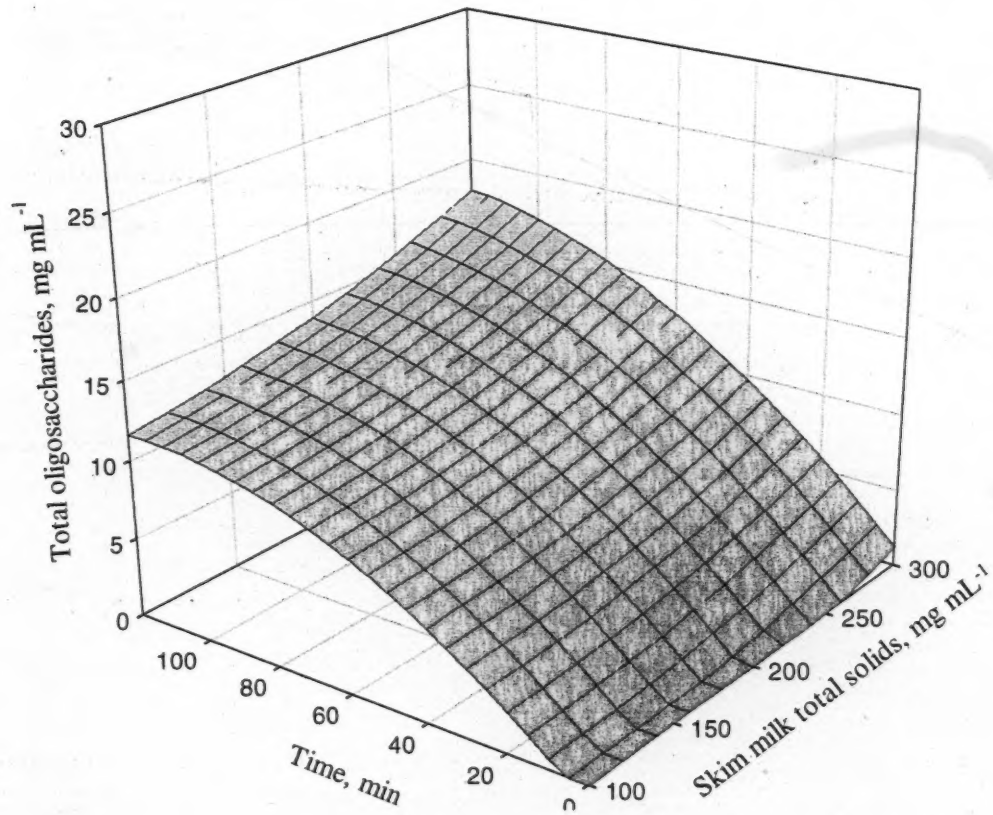

Figure A-11: Predicted oligosaccharide formation during the lactose hydrolysis in skim milk systems by *Streptococcus thermophilus* 143 CCE at 50°C

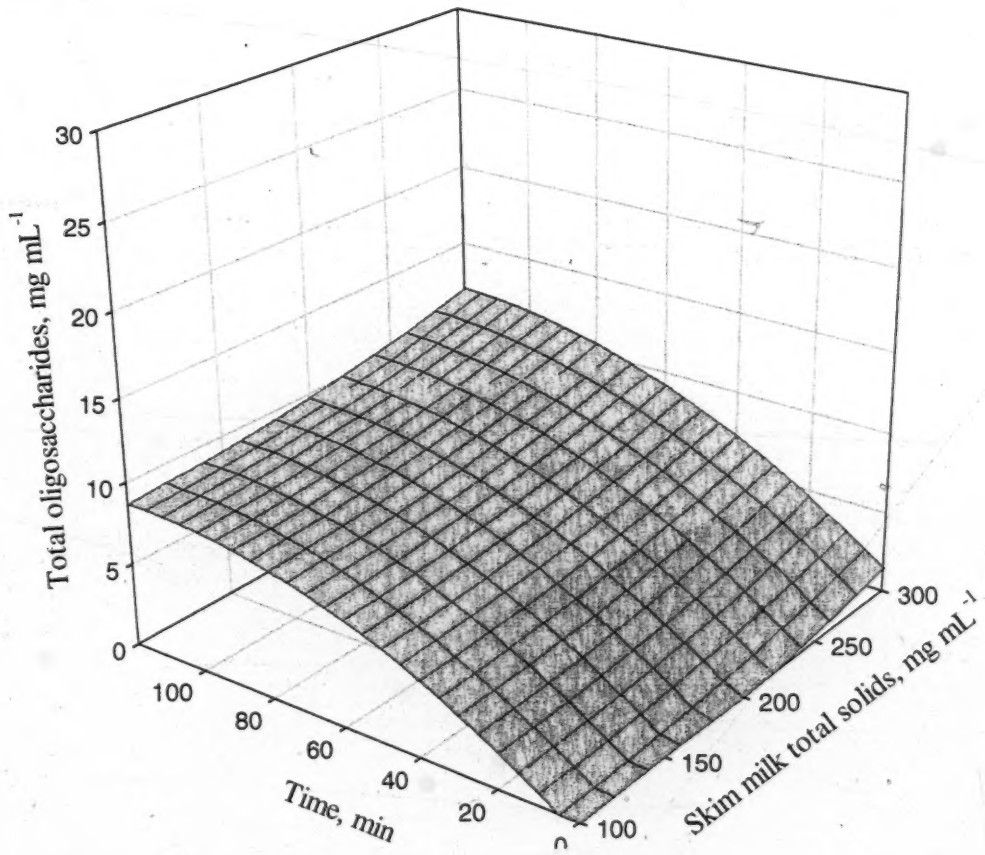

Figure A-12: Predicted oligosaccharide formation during the lactose hydrolysis in skim milk systems by *Lactobacillus delbrueckii* ssp. *lactis* 3078 CCE at 50°C

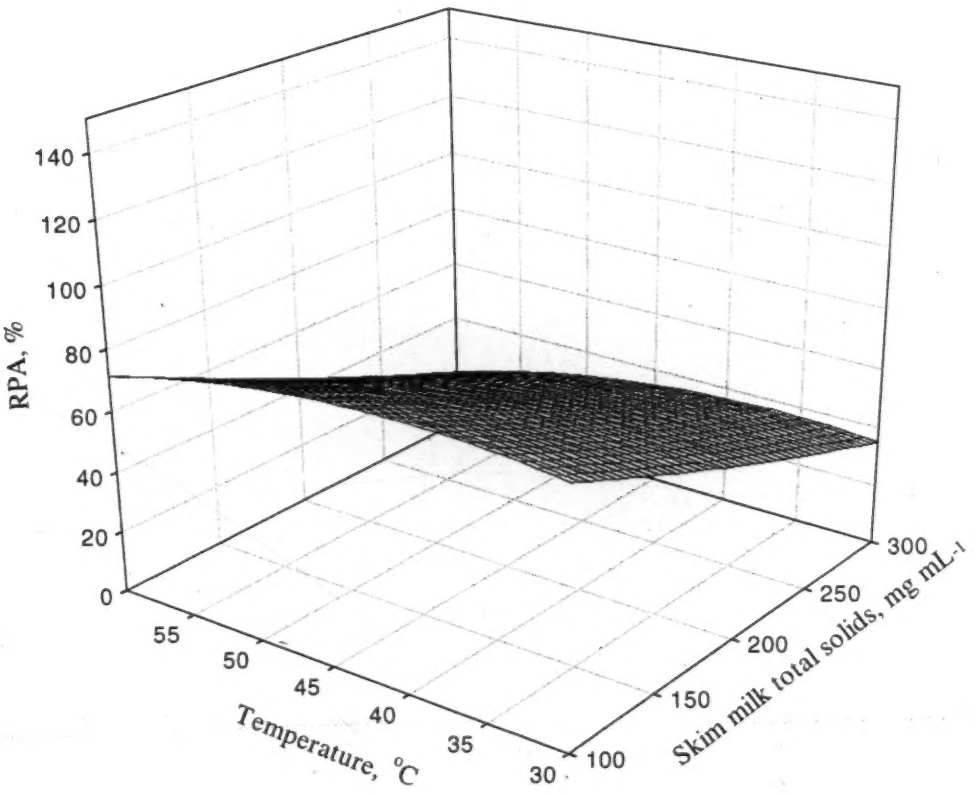

Figure A-13: Predicted relative proteolytic activity (RPA, %) during the lactose hydrolysis by *Streptococcus thermophilus* 143 CCE in skim milk systems at the end of 120 min reaction period

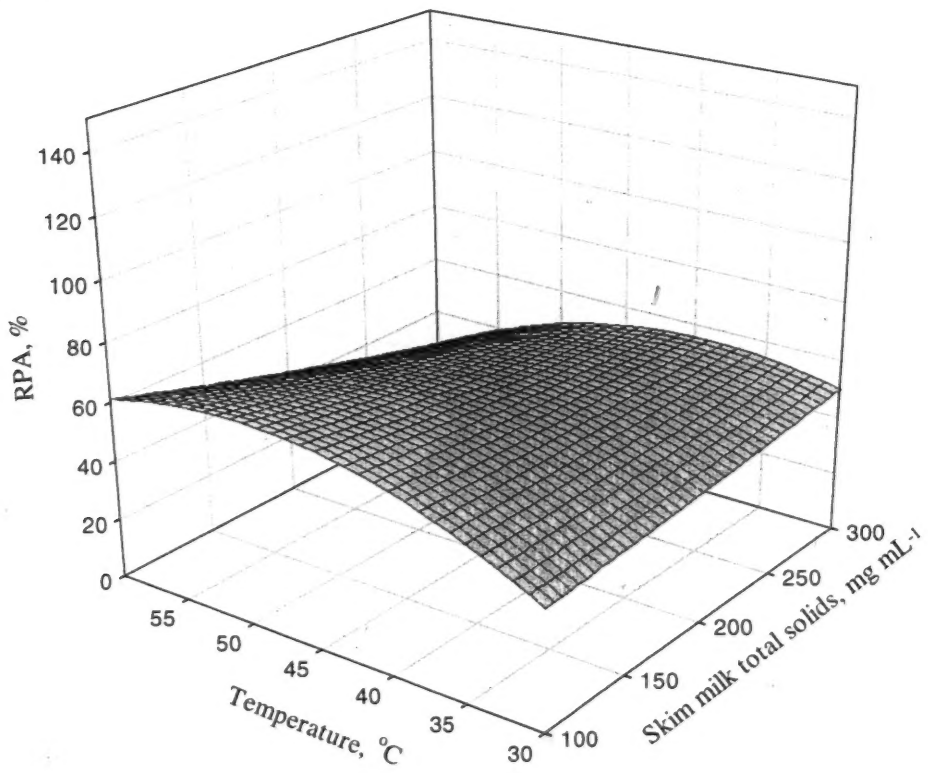

Figure A-14: Predicted relative proteolytic activity (RPA, %) during the lactose hydrolysis by *Lactobacillus delbrueckii* ssp. *lactis* 3078 CCE in skim milk systems at the end of 120 min reaction period

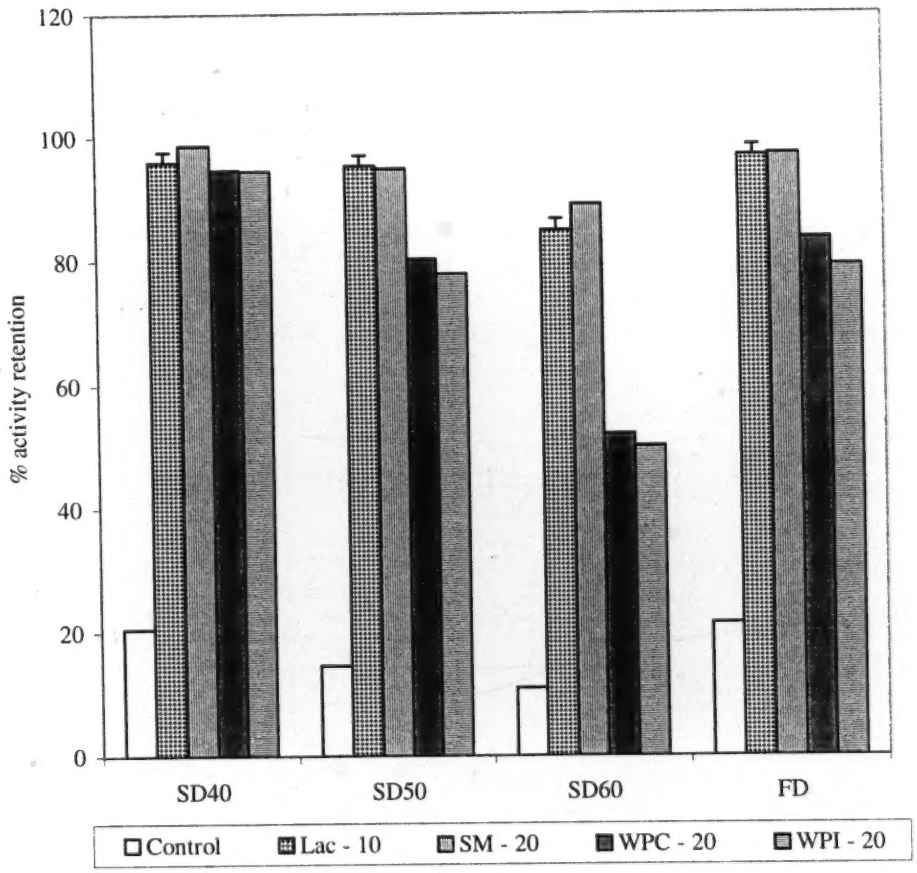

Figure A-15: The effect of drying methods on the β-galactosidase activity retention in CCE powders from *Lb. bulgaricus* 11842 upon addition of 10% (w/v) of dairy drying adjuncts.

(Control – CCE in skim milk salt buffer (SMSB); Lactose (Lac), WPC and WPI – 10% final concentration (w/v) in SMSB; SM – 20% final concentration of skim milk solids (w/v) in deionized water, included based on approx. 10% lactose content; Bars present the adjusted standard error of the mean, ±1.695).

Table A-3: Moisture content of dry powders containing crude β-galactosidase extracts from *Lb. bulgaricus* 11842 obtained by addition of intermediate concentrations of drying adjuncts

Treatment*	Moisture content, %			Water activity	
	Initial	15 days	30 days	Initial	30 days
SD 40					
Lac - 10	5.0	6.5	9.4	0.303	0.415
SM - 20	5.8	6.1	6.9	0.281	0.398
WPC - 20	5.3	6.3	7.8	0.240	0.383
WPI - 20	5.0	5.6	6.3	0.250	0.350
SD 50					
Lac - 10	4.4	5.2	6.4	0.287	0.347
SM - 20	4.9	5.5	6.6	0.297	0.357
WPC - 20	4.5	5.8	7.1	0.241	0.331
WPI - 20	4.2	5.0	6.4	0.259	0.349
SD 60					
Lac - 10	3.3	3.8	4.5	0.250	0.310
SM - 20	3.7	4.0	4.5	0.270	0.330
WPC - 20	3.6	5.4	7.1	0.213	0.283
WPI - 20	3.3	3.9	4.9	0.208	0.278
FD					
Lac - 10	3.7	5.5	8.1	0.278	0.333
SM - 20	1.3	3.7	6.3	0.126	0.176
WPC - 20	0.7	1.4	3.3	0.046	0.214
WPI - 20	0.5	1.6	3.5	0.034	0.263
SEM		0.41		0.016	

***SD and FD – spray and freeze drying, respectively; Lac, SM, WPC and WPI – lactose, skim milk total solids, whey protein concentrate and isolate in indicated concentrations (w/w); SEM – standard error of the mean.**